Report to the National Aeronautics and Space Administration

"Cytochemical Studies of Planetary Microorganisms - Explorations in Exobiology"

NsG 81-60

Status Report Covering Period October 1, 1967 to April 1, 1968

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A. INTRODUCTION

This Status Report covers the activities of the Instrumentation Research Laboratory from October 1, 1967 to April 1, 1968. Major technical efforts are described in separate technical reports and papers. The status report refers to these and summarizes continuing projects.

Work under grant NsG 81 includes areas of research that are closely related to efforts being carried out in the Department of Genetics under other grants or contracts. The Air Force Contract AF 49(638)1599 for "Molecular Biology Applications of Mass Spectroscopy" was completed on December 31, 1967. Other grants are National Institute of Neurological Diseases and Blindness Grant NB-04270 entitled "Molecular Neurobiology" and work carried out by the Advanced Computer for Medical Research (ACME) program supported by the National Institutes of Health, Division of Research Facilities and Resources under Grant FR00311-01. There is collaboration with the work in the Computer Science Department on artificial intelligence carried out under support of the Advanced Research Projects Agency SD 183. In addition, work is being done on "Genetic Studies of Mammalian Cells", National Institutes of Health, under Grant CA04681-08. The relationship of the work carried out under this NASA grant to these other activities continues to prove of great mutual benefit in all cases.

The general project areas of the program resume, part 3 of the status report are:

I. Gas Chromatography and Optical Resolution
II. Mass Spectrometry
III. Computer Managed Instrumentation
IV. Cell Separator

These projects continue to contribute to technical mastery of problems in exobiology by furnishing specific analytical techniques of high sensitivity and discrimination for the detection of exotic life. Our current efforts, in collaboration with the NIH funded ACME program, have allowed us to learn a great deal and will permit us to learn more about computer-controlled instruments, using ACME as a system prototype of an Automated Biological Laboratory (ABL). The problem of providing a time-shared computer system for high data rate, sporadic demand laboratory users has proved to be more difficult than we had originally envisaged. While some of the problems can be traced to hardware limitations, the main difficulties center on the production of very complex but reliable software for the operating system. The ultimate demonstration of the feasibility of a computer-controlled automated biological laboratory will depend on the availability of resources for a great deal of system research and development and dedicated computer facilities.

During the six month period described above, seven papers were submitted to journals for publication, in addition to those of Professor Djerassi's laboratory, and three technical reports prepared. A listing of these papers and reports is included in this status report.

B. PROGRAM RESUME

I. Gas Chromatography and Optical Resolution

A. Gas Chromatography of Amino Acids

Work on the quantitative gas liquid chromatographic analysis of amino acids has been continued. The analysis is based on the conversion of the amino acids to the stable N-thiocarbonyl alkyl esters, which with the exception of arginine, can be gas chromatographed. The reaction sequence used is esterification of the amino acid mixture, followed by a treatment with carbon disulfide and triethylamine to yield a dithiocarbamate. This on treatment with a chloroformate ester gives an unstable carboalkoxy dithiocarbamate which decomposes to the thiocarbonyl derivatives. It has now been established, that quantitative work with a reproducibility of 5% can be carried out with synthetic mixtures containing 0.2 μM of each protein amino acid. Ribonuclease (A) hydrolysates (0.2-0.5 mg) which were carried through the entire chemical and chromatographic procedure also gave clean and readily interpretable chromatograms. Although chemical manipulation requires 2 1/2 hours, instrument analysis time has been reduced to 1 hour. With our present equipment 6-8 analyses can be done during an 8 hour day, which compares favorably with the 2-3 analysis capability of our amino acid analyzer.

B. Factors Affecting the Separation of Diastereoisomeric Compounds by G.L.C.

Gas liquid chromatography (g.l.c.) of diastereoisomers has been used with great success for the determination of steric purity of asymmetric compounds, but no rationale has been proposed for the selection of suitable resolving agents and chromatographic conditions, for the application of such techniques. Earlier work on the mechanism of g.l.c.

Table I

Gas Chromatographic Separation of Diastereoisomeric N-Trifluoroacetyl-S-Phenylalanine-(I)alkyl Esters

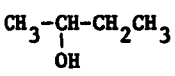
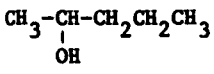
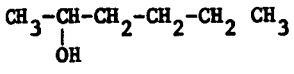
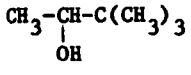
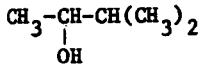
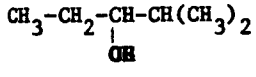
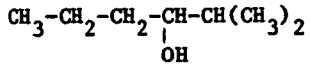
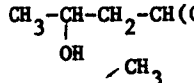
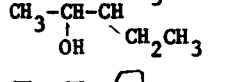
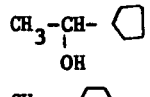
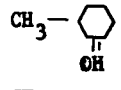
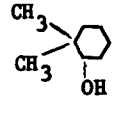
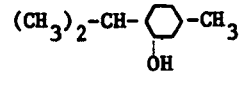
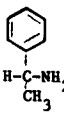
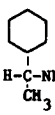
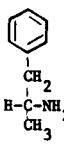
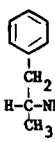
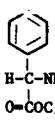
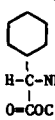
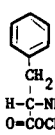
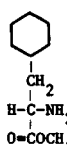
Compound No.	Structure of Asymmetric Alcohol	Separation Temperature °C	Retention Time Of Diastereoisomers (Min.) S-(-)	S-(+)	Ratio of Separation Temperature α
I		138	15.30	15.90	1.04
II		138	19.40	19.80	1.02
III		138	25.50	26.30	1.03
IV		155	9.00	10.30	1.17
V		155	8.50	9.20	1.10
VI		155	10.60	10.90	1.03
VII		155	11.70	12.00	1.03
VIII		155	9.20	9.40	1.03
IX		155	11.00	11.70	1.07
X		170	12.50	13.70	1.11
XI		170	13.40	14.60	1.10
XII		170	14.20	15.80	1.12
XIII		170	16.70	19.40	1.18

Table II

Gas-chromatographic Separation of Diastereoisomeric TFA-S-prolylamides Derived from Asymmetric Amines and Amino Acid Esters.

Asymmetric Compounds	Compound Number	Structure	Column	Separation Temperature (°C)	Retention Time of Diastereoisomers (Min.) SR	SS	Ratio of Retention Times $\alpha = \frac{SS}{SR}$
1-amino-1-phenylethane	XIV		5% QF-1	210	3.80	4.50	1.18
			0.5% EGA	180	5.90	8.00	1.36
1-amino-1-cyclohexyl-ethane	XV		5% QF-1	210	3.70	4.15	1.12
			0.5% EGA	180	2.70	3.35	1.24
Amphetamine	XVI		5%QF-1	210	5.15	5.90	1.08
			0.5% EGA	200	3.10	3.80	1.23
Desoxyephedrine	XVII		5% QF-1	210	8.80	9.80	1.12
			0.5% EGA	200	4.80	5.30	1.10
Phenylglycine ethyl esters	XVIII		5% QF-1	210	10.20	10.20	1.00[a]
			0.5% EGA	200	7.90	8.70	1.10
Cyclohexylglycine ethyl ester			5% QF-1	210	10.10	10.90	1.08
			0.5% EGA	200	4.20	4.80	1.16
Phenylalanine methyl ester	XX		5% QF-1	210	12.00	13.00	1.08
			0.5% EGA	200	10.40	10.70	1.03
Cyclohexylalanine methyl ester	XXI		5% QF-1	210	10.25	10.90	1.06
			0.5% EGA	200	5.10	5.80	1.14

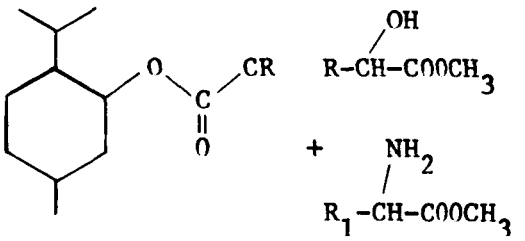
separation of diastereoisomers had suggested that the degree of branching of a substituent at one asymmetric center and the distance between the optical centers are important variables in determining separation efficiencies. Since knowledge of the factors which influence the g.l.c. resolution of diastereoisomers would help in the choice of a resolving agent, we have now examined the effect of some systematic structure variations on the degree of separation of diastereoisomeric esters and amides. An examination of the data on Table I and II, suggests that branching of substituents and the contrast between the atomic numbers of the groups attached to one of the asymmetric centers are factors influencing separation. The degree of resolution of diastereoisomeric amides is also affected by solute-solvent interactions involving primary amide bonds or aromatic rings with polyester stationary phases (Table II). Our results suggest that a resolving agent should be a readily available, low molecular weight, optically pure compound containing a suitable functional group which is close to the asymmetric center. The reagent should contain groups of widely different atomic numbers around the asymmetric center and incorporate a secondary or tertiary carbon attached directly to the asymmetric atom. Alternatively, planar cyclic compounds with a functional group adjacent to the asymmetric center also make excellent resolving agents.

C. Menthyl chloroformate, a new agent for the gas chromatographic resolution of asymmetric alcohols, amines, amino-acids and hydroxy-acids.

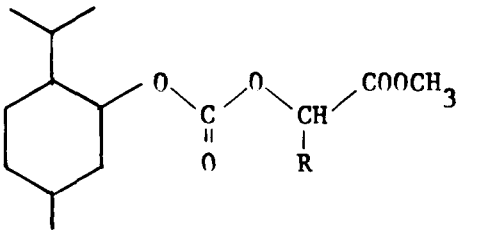
In a recent review on cyclodepsipeptides,[1] it was pointed out that a GLC method of determining simultaneously the configuration of amino-acids and hydroxy-acids would greatly decrease the amount of natural product necessary for structure determination. Although our N-TFA-S

[1] L. D. W. Russell, Quart. Rev., 21, 559 (1967)

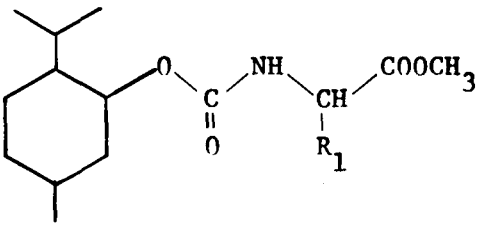
prolyl chloride reacts well with amino acids it does not react readily with α-hydroxy acids. We have now found that (-)-menthyl chloroformate (I)[2] reacts with both α-hydroxy acids and α-amino acid methyl esters to give the corresponding diastereoisomeric carbonates (II) and urethanes (III) which are resolvable by GLC (Table III).



I



II



III

The ease with which (-)-menthyl chloroformate reacted with the α-hydroxy acids suggested that it could also be used for the optical analysis of α-alkyl benzyl alcohols which are not amenable to the esterification procedure used for the alkanols.[3] It was found (Table IV) that the resulting carbonates could be resolved by gas chromatography and in addition, some amines of known configuration were also analyzed by this technique.

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[2] L. A. Carbino, Chem. Comm., 23, 858 (1966)

[3] B. Halpern and J. W. Westley, Aust. J. Chem. 19, 19533 (1966).

TABLE III

Gas chromatographic separation of α-hydroxy and α-amino acid methylesters as their R-(-)-menthoxycarbonyl diastereoisomeric derivatives.*

Acid	Separation Temperature (°C.)	Retention time of diastereoisomers (min.) SR	SS	Ratio of retention times $\frac{SS}{SR}$
Lactic	170	3.85	4.2	1.09
α-Hydroxyisovaleric	170	5.00	5.7	1.14
α-Hydroxyisocaproic	170	6.8	7.8	1.15
3-Phenyllactic	200	8.5	9.7	1.14
Alanine	170	6.6	7.0	1.06
Valine	170	9.05	10.0	1.10
Leucine	170	11.9	12.75	1.07
Phenylalanine	200	13.1	14.4	1.10

*GLC analyses were carried out on 5 ft. x 1/8 in column packed with 5% QF-1 on Aeropak 30. The nitrogen flow during analyses was 30 ml/min.

TABLE IV

GLC separation of α-alkyl benzyl alcohols and α-alkyl aryl amines as their R-(-)-menthoxycarbonyl diastereoisomer derivatives*

	Column	Temp.	Retention time of Diastereoisomers RS	RR	Ratio of Retention times (α)
1-phenyl-1-ethanol	A	170°	5.3	5.9	1.11
1-phenyl-1-propanol	A	170°	5.5	6.2	1.13
1-phenyl-1-butanol	A	170°	6.8	7.6	1.12
1-phenyl-1-pentanol	A	170°	8.0	8.9	1.11
1-(p-tolyl)-1-butanol	A	170°	8.1	9.3	1.15
1-phenyl-1-cyclohexyl carbinol	A	200°	6.45	7.0	1.09
1-phenyl-1-ethylamine	B	180°	9.5	10.1	1.06
1-(1-naphthyl)-1-ethylamine	B	230°	6.6	7.0	1.06

*GLC analyses were carried out on 5' x 1/8" columns with a nitrogen flow of 30 ml./min.

Column A: 3% EGS on AEROPAK 30.

Column B: 5% QF-1 on AEROPAK 30.

II. Mass Spectrometry

A. Analysis of Natural Products

Professor Djerassi's laboratory in the Department of Chemistry has yielded the results reported in the following papers:

Djerassi, C.; Jackson, A. N.; Kenner, G. W.; Budzikiewicz and Wilson, J. M.: Mass Spectrometry in Structural and Stereochemical Problems XC. Mass Spectra of Linear Di-, Tri- and Tetrapyrrolic Compounds. Tetrahedron, 23, 603 (1967).

MacLeod, J. K.; Thomson, J. B.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXXII. Possible Operation of Tautomerism Before and After Electron-Impact Induced Fragmentation of Molecular Ions. Tetrahedron, 23 2095 (1967).

Brown, P.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXXX. A Study of Electron Impact Induced Migratory Aptitudes. J. Am. Chem. Soc., 89, 2711 (1967).

Reusch, W.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXVII. Aliphatic α,β-Epoxyketones. Tetrahedron, 23, 2893 (1967).

Buchardt, O.; Lohse, C.; Duffield, A. M.; Djerassi, C.: The Photolysis of 1-Phenyl and 1-Cyano Substituted Isoquinoline N-oxides to Benz [f]-1,3-Oxazepines. Tetrahedron Letters, 2741 (1967).

Harris, R. L. N.; Komitsky, F., Jr.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXXXII. Electron-Impact Induced Alkyl and Aryl Rearrangements in α,β-Unsaturated Cyclic Ketones. J. Am. Chem. Soc., 89, 4765 (1967).

Harris, R. L. N.; Komitsky, Jr. F.; Djerassi, C. Mass Spectrometry in Structural and Stereochemical Problems CXXXIV. Electron Impact Induced Alkyl and Aryl Rearrangements in α-Arylidene Cyclic Ketones. J. Am. Chem. Soc., 89, 4775 (1967).

MacLeod, J. K.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXXXVI. Primary Hydrogen Isotope Effects in the McLafferty Rearrangement. J. Am. Chem. Soc., 89, 5182 (1967).

Green, M. M.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXXXVII. Examples of Interaction of Remote Functional Groups after Electron Impact. J. Am. Chem. Soc., 89, 5190 (1967).

Carpenter, W.; Duffield, A. M.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXLVII. The Course of the Electron Impact Induced Elimination of Hydrogen Fluoride in Primary Alkyl Fluorides. Chem. Comm., 1022 (1967).

MacLeod, J. K.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXL. Competitive McLafferty Rearrangements in Bifunctional Compounds. J. Org. Chem., 32, 3485 (1967).

Carpenter, W.; Duffield, A. M.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXLIII. Unusual Fragmentations in the Mass Spectra of Some Aliphatic Ethers. J. Am. Chem. Soc., 89, 6164 (1967).

Carpenter, W.; Duffield, A. M.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXLV. Factors Governing the Preferential Loss of Small versus Large Radicals in Ketones, Schiff Bases and Ethers. J. Am. Chem. Soc., 89, 6167 (1967).

Duffield, A. M.; Djerassi, C.; Sandstrom, J.: Mass Spectrometry in Structural and Stereochemical Problems CXXXVIII. Fragmentation Processes of Some Thiocarbonyl Compounds on Electron Impact. Acta Chem. Scand., 21, 2167 (1967).

Carpenter, W.; Duffield, A. M.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXLIV. Unusual Fragmentations of the Low-Voltage Spectra of Aliphatic Ketones. J. Am. Chem. Soc., 90, 160 (1968).

Sengupta, P.; Chakraborty, A. K.; Duffield, A. M.; Durham, L. J.; Djerassi, C.: Terpenoids LXI. The Structure of a New Triterpene, Putranjivadione. Tetrahedron, 24, 1205 (1968).

Schroll, G.; Lawesson, S.-O.; Duffield, A. M.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXXXIX. The Fragmentation of Some Cyanohydrins and O-Acyl-cyanohydrins Upon Electron Impact. Arkiv Kemi, 28, 435 (1968).

Carpenter, W., Sheikh, Y. M.; Duffield, A. M.; Djerassi, C.; Mass Spectrometry in Structural and Stereochemical Problems CLIII. Electron Impact Promoted Fragmentation of N-Alkyl Cyanides. Org. Mass Spectr., 1, 3 (1968).

Briggs, W. Scott; Djerassi, C.: The Pyrolysis of the Epimeric d_0 - and 2-d_1-2-Methylcyclohexyl-S-Methyl Xanthates and Acetates. J. Org. Chem., 33, 1625 (1968).

Duffield, A. M.; Djerassi, C.; Mazerolles, P.; Dubac, J.; Manuel, G.: Mass Spectrometry in Structural and Stereochemical Problems CLII. Electron Impact Promoted Fragmentation of Some Substituted Germacyclopentanes. J. Organometallic Chem., 12, 123 (1968).

Brown, P.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CXLVIII. Electron Impact Induced Fragmentations of Cyclic Sulfites and Carbonates. Tetrahedron, 24, 2949 (1968).

Duffield, A. M.: Mass Spectrometric Fragmentation of Some Lignons. J. Heterocyclic Chem., 4, 16 (1967).

B. Mass Spectral Microanalysis of Organic Solids.

The pulsed ruby laser and optical system described in Technical Report No. IRL 1061 has been used to vaporize organic samples in the source chamber of our Bendix time-of-flight mass spectrometer. It was noted in the previous report that the laser and entire optical train are external to the vacuum environment of the mass spectrometer source and analyzer chamber. The laser radiation condensed by the terminal lens in the optical system is directed through a plane Corning 7052 glass window to the target. The window contributes spherical aberration. At maximum laser output and full aperture at the condensing lens, we produce approximately 70μ diameter spots at the target. The spot size can be reduced either by diminishing the laser output, which reduces the beam spread, or by stopping down the condensing lens, which reduces the spherical aberration. We are able to work down to about 35μ diameter spot size by either of these procedures.

Most of our work to date has been performed on individual grains and grain clumps from powdered samples. We ran a sensitivity test with various sizes of target clumps of Bromouracil. The mass spectral signal generally sank into the noise at clump diameters below 10μ.

Though the process of laser vaporization results in the generation of some ions, we do not rely on these ions for mass analysis. They tend to become too widely dispersed along the direction of the flight axis of the instrument to be focused properly. Thus, in both the crucible and laser modes we rely on electron beam ionization of neutrals for ion production.

We have developed a practice of storing all of our data in the Stanford Medical Center IBM-360 ACME time-shared computer system. The crucible runs are slow enough to permit direct tape recording and transmission

to ACME via a local INC computer. The laser data must be manually transcribed from photographs of oscilloscope display of the output.

We have been interested in experimenting with the visual comparison of spectra. For this purpose we have written a program to display pairs of spectra in a comparative format on a television unit driven by the computer. The basic display format that appears when the program is first called is illustrated in Fig. 1, which is reproduced from a Polaroid photograph of the television screen.

The image consists of a square pattern divided into a set of rectangular zones. The horizontal line 16 is the common base line for two spectra that may be simultaneously displayed. The mass peaks for the spectrum displayed in region 4 will extend upward from the base line and for the spectrum displayed in region 5 they will extend downward. For each of the two line spectra the mass numbers increase to the right. The numbers in zones 6 and 8 denote the lower and upper limits, respectively, of the mass range for the upper spectrum. The numbers in zones 7 and 9 serve a similar function for the lower spectrum. The numbers in zones 2 and 3 identify the record numbers of the upper and lower spectra, respectively.

All control in execution is accomplished by the use of a deck mounted gearshift-like control with a sensing button on the end of it. For historical reasons, this unit and the blinking spot 1 that it positions on the display are referred to as "mouse". The horizontal and vertical coordinates of the spot 1 on the screen are sensed by potentiometers coupled to the manual control. These coordinates are read upon release of the depressed sensing button.

When it is desired to select or change either a spectrum record number or the mass limits, mouse is moved into the area consisting of the set of zones 10, numbered 0 through 9. A multiple digit number collected

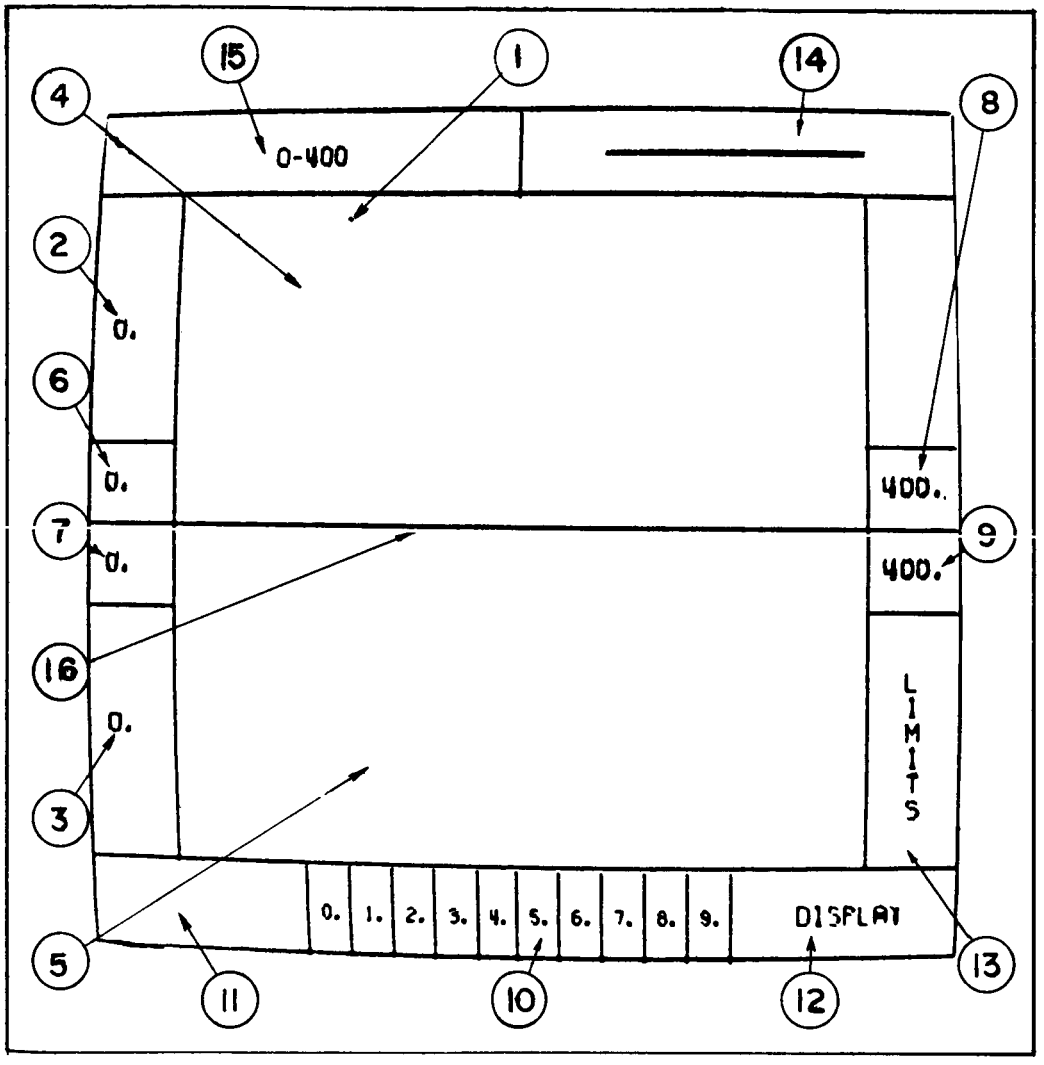
15
1
14
4
8
0-400
2
0.
6
0.
400.
7
0.
400.
9
16
0.
LIMITS
3
0. 1. 2. 3. 4. 5. 6. 7. 8. 9.
DISPLAY
5
13
11
10
12

FIGURE 1

from region 10 by successive selections is automatically accumulated in zone 11 for checking. The flashing of bar 14 advises the user that the computer is waiting for mouse. This information is useful when the computer is in heavy use. The selected number may be either corrected or transferred to any one of zones 2, 3, 6, 7, 8, or 9 by directing mouse to the desired zone and releasing the button. After selection is made, mouse may be used to sense zone 12, labeled DISPLAY, thereby signaling the computer to display the selected spectra over the chosen mass ranges. The photograph reproduced in Figure 2 demonstrates the comparison of the base guanine obtained by use of the laser, record 31 displayed up, and the guanine spectrum obtained by use of the crucible, record 35 shown down. Sensing mouse above the base line and at the vicinity of any mass peak causes the V-like symbol 17 to appear at the mass peak and the value of the mass,peak, 151 in this case, to be displayed at location 18. Mass 151 is the guanine molecular peak. Sensing below the base line causes the inverted V denoted 19 (not reproduced clearly here) to appear at the mass peak and the associated mass number 202 to appear at location 20. Mass 202 is one of the mercury isotopes arising from the use of mercury as a pump fluid.

The photograph reproduced in Figure 3 illustrates some of the options available to the user. Record 37, salmon sperm DNA volatilized by the laser system, is displayed over the mass range 0-400 in the upper spectrum. The lower spectrum is a portion of the same spectrum showing in further detail the masses ranging from 100 to 150. The base peaks for cytosine (mass 111), thymine (mass 126) and adenine (mass 135) are present, indicating that these substituants are detached as recognizable fragments from the macromolecule. Guanine got lost in the noise on this particular shot; but has been seen on others.

Figure 4 illustrates the manner in which the display shown in Figure 3 was obtained. Spectrum 37 in the mass range 0-400 was first displayed both up and down. Mouse was then sensed in region 13, labeled LIMITS.

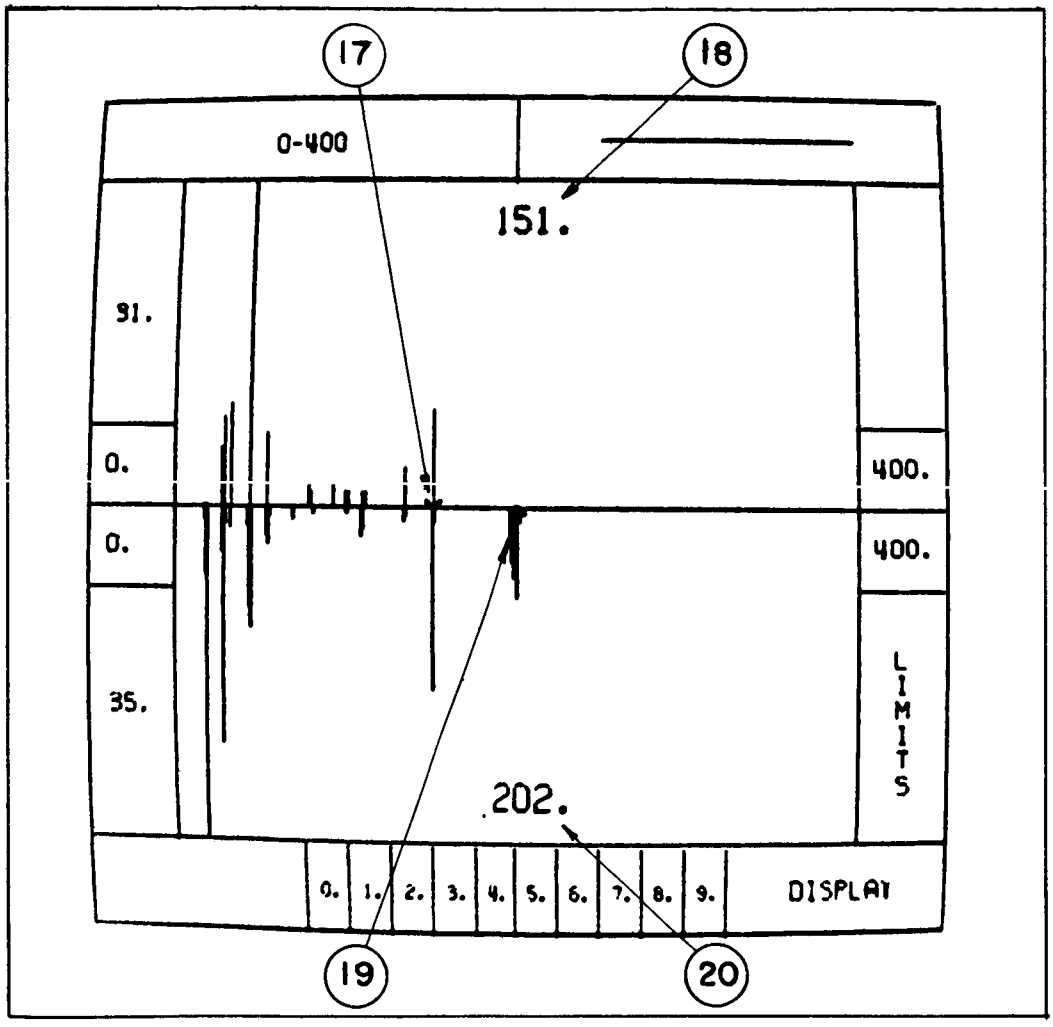
17
18
0-400
151.
91.
0.
0.
35.
400.
400.
LIMITS
202.
0.
1.
2.
3.
4.
5.
6.
7.
8.
9.
DISPLAY
19
20

FIGURE 2

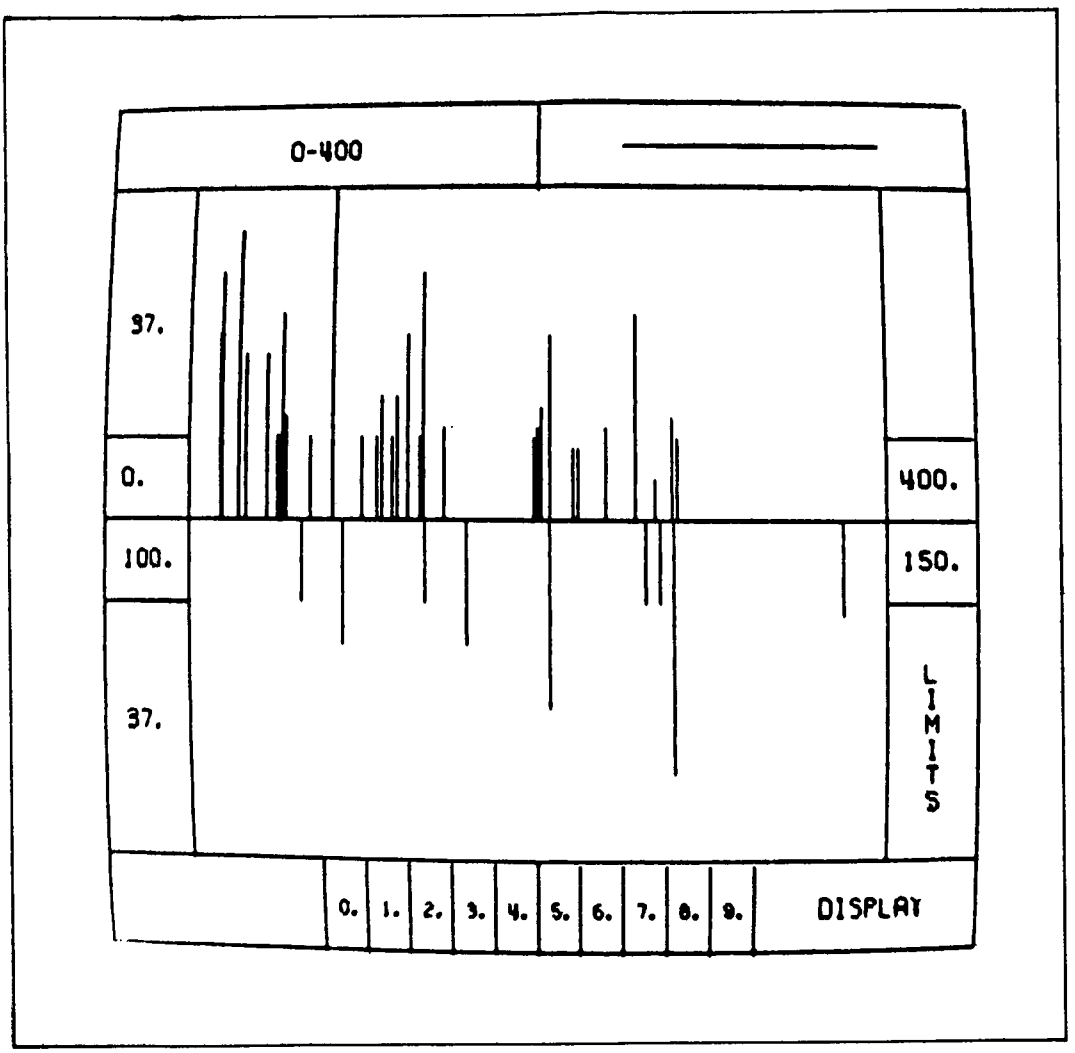

FIGURE 3

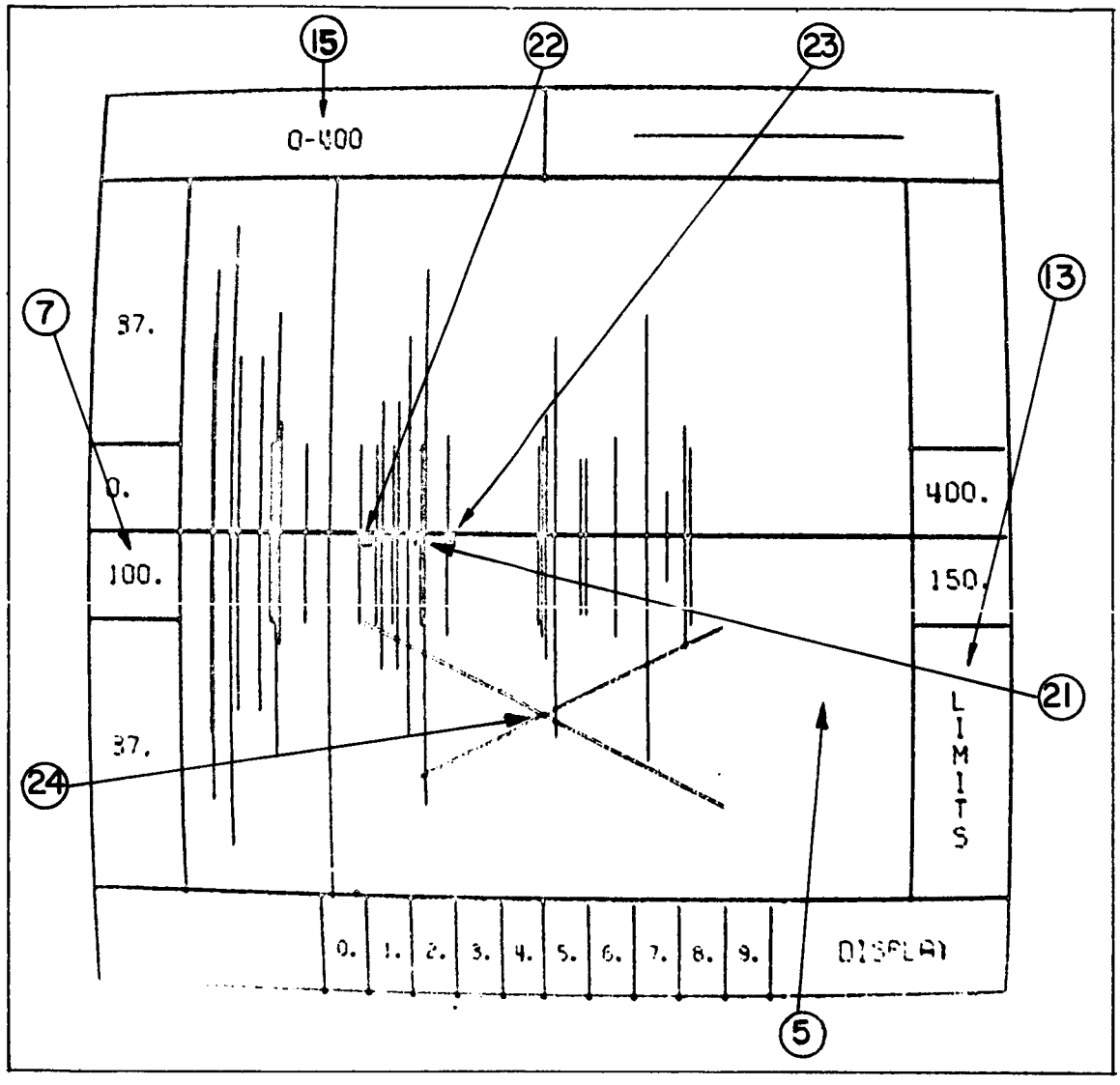

FIGURE 4

Following the sensing of LIMITS, mouse was sensed in zone 5 at that mass position intended to be an upper limit for the lower bound on the desired expanded mass range. An enhanced bar such as the one shown at 21 then appeared at the position designated by mouse. Sensing mouse in the lower limit zone 7 then transferred the newly selected lower limit to this zone. Because of the discreteness of available point sites on the digitally driven television screen the lower limit is automatically rounded to the nearest multiple of 25 less than or equal to the mass identified by the enhanced bar. Mass 100 resulted. An L-like symbol, denoted 22, is created to show the mass location of the new lower bound on the display being generated. A flashing X appears at 24 to tell the user that the displayed spectrum is no longer valid. Sensing mouse successively in the LIMITS zone, in the mass zone, and in the high limit zone provides the new upper limit 150.

Sensing DISPLAY then results in the appearance of display shown in Figure 3. If mouse is subsequently sensed in zone 15, labeled "0-400", the upper and lower limits of either of the spectra may be restored to the range 0-400 by a subsequent sensing of mouse either above or below the central base line of the display.

Table V lists the spectra that have been obtained and are available in the computer data file for display and comparative analysis.

III. Computer Managed Instrumentation

A. ACME

ACME system programmers have converted the IBM 1800 control program to have time-sharing capability. Previously only the IBM 360/50 was time shared; the IBM 1800 was used in single user mode only.

TABLE V

Sample	Crucible	Laser
B-Subtilus - DNA	x	
Salmon Sperm DNA	x	x
Adenylic Acid	x	
Deoxycytidine	x	x
Deoxyguanosine	x	x
Deoxyadenosine	x	x
Deoxyadenocine Monophosphate		x
Deoxyguanosine Monophosphate		x
Thymidine Phosphate	x	x
Thymine	x	x
Cytosine	x	x
Guanine	x	x
Adenine	x	x
Bromouracil	x	x
P_2O_5	x	x
Dextrose		x
Oxydized Copper Probe		x
Tatooed Skin		x
Untatooed Skin		x
Fezan Black		x
Alanine		x
Isoleucine		x
Cysteine		x

The time-shared program should obviate much of the scheduling problems inherent under the old system while allowing the users greater flexibility in other respects.

Previously each user (remote laboratory connections) was limited to six input lines, with only one of them digital. Now he may use 12, with any ratio of digital and analog. Where previously all the input lines were sampled at the same rate for the same duration, the present system allows the user to sample different input lines at different rates, for various amounts of time.

The parameters for user's buffer size, sample rate, and line number are stored in a 360 ACME systems program and may be altered by the user. Previously, it was difficult to vary these parameters "on-line" or during execution of the program. Two additional features are currently being worked on. Presently a start interrupt is used to trigger all analog input lines and all digital input lines. In the future the user will be able to have different start interrupts on different input lines. Currently, digital output is not available to most of the 1800 users. This output function will be implemented in the near future.

These, and the features reported in the last status report, have been demonstrated. However as a day to day operation, users have had to sign up for 1800 time and unique user-parameter cards and fed into the 1800 manually for each application.

B. 270X-270Y ACME Computer Connections.

The 270X-Y system is a specially built IBM digital interface for the time sharing ACME system. This was partially paid for by the NASA Grant NsG 81. The 270X is the central terminal that is housed in the main computer room and the 270Y's are the remote (up to 5000 feet) terminals designed for individual laboratories. Their purpose is to

allow the transmission of laboratory generated or required digital data between the central processor of the IBM 360/50 and the laboratory. This is in a time shared mode under control of the program written and executed in the time shared ACME system. It was specifically designed for high data rates.

The 270X-Y's were installed during the last reporting period (see page 21 Technical Report IRL 1061). During December 1967 of this reporting period the 270X-Y's did pass the acceptance tests mutually agreed to by Stanford and IBM. An attempt was made to have the conditions of the tests resemble those of anticipated 270Y use. However the tests were designed to determine the acceptability of the 270X-Y and not especially the entire related ACME system.

Since then a number of problems have developed in attempting to apply the 270Y's to sophisticated uses. First, there seems to be an interaction between the 270X-Y and the ACME system (software and hardware) that causes catastrophic failure of the ACME time sharing program. When this happens, a computer failure occurs that affects all on-line time sharing users. It is necessary to reinstate the entire system, a procedure of 5 to 15 minutes, to recover. Each user must then re-enter his own program. To minimize such occurrences, until the design difficulty is resolved, the Instrumentation Research Laboratory has limited the development of new uses to a restricted schedule of hours.

Second, unanticipated channel restraints were found in the ACME (software) system that operates the 270X-Y. ACME uses a combination of the ACME developed software and the IBM (software) operating system. The 270X-Y can respond at a high speed (though this might evoke a failure of the kind mentioned above) during transfers of strings of digital data. Such strings are limited to approximately 64,000 bytes. The response time from one string to another is from 0.05 seconds to a few seconds. Furthermore a "read" and a "write" are separate and sequential strings.

Thus, presently, the following important limitations arise:

1. Long (over 64,000) high speed data transfers are not possible.
2. Fast (response times of 1 or 2 milliseconds) Write (control) - Read (data) is not possible.
3. Fast (response times of less than seconds) Read (data) - Computer logical decisions - Write (control) interaction is not possible.

These restraints have caused delays in the mass spectrometer instrumentation programs. This is reported in greater detail in following portions of this report.

The unexpected delays in perfecting this data-oriented time-shared system point to the need for extensive system research before the Automated Biological Laboratory can be committed to this approach.

Personnel from the affected groups (IBM, IRL and ACME) are attempting to find solutions or means of use that will avoid the difficulties. Nevertheless, progress has been made in the use of the 270X-Y's.

Two of the 270Y's have been equipped with IRL designed "multiplexer" units. These allow the user to select any one of the four sets of screw down input/output buses in addition to the set of panel jacks supplied by the manufacturer. Also provided is a reference voltage system for each piece of equipment connected. Thus one 270Y can be used with any one of several pieces of equipment without reference voltage considerations and the cumbersome transfer of twenty panel jacks.

Software has been developed (in conjunction with the ACME staff) to utilize the 270X-270Y hardware for on-line digital plotting, controlled by the ACME time-shared system. This vastly increases the usability of the Calcomp plotters over the previously reported user modes.

This plotting capability has proven valuable in presenting the results of computations performed on the output of the Dichroscope. The program provides for a rather conventional time shared data storage and retrieval function and also provides for the time shared plotting of the results of certain computations.

C. Mass Spectrometer-Computer Instrumentation

The Instrumentation Research Laboratory has a number of concurrent projects concerning mass spectrometers. The mass spectrometers involved are:

1. A Bendix Time-of-Flight located in the main electronics laboratory on the ground floor.
2. A Finnigan 1015 quadrupole model 1015 on the third floor in the exobiology laboratory.
3. A high resolution AEI MS-9 in the Chemistry Department some 1500 feet away.
4. An Atlas CH-4 single focusing magnetic instrument also in the Chemistry Department.

Instrumentation is being developed using three computers:

1. LINC, a 2K 8 microsecond machine with magnetic tapes. This is located in the Instrumentation Research Laboratory.
2. ACME's 360/50 used in a time shared mode. This is housed on the floor above the Instrumentation Research Laboratory.
3. IBM 1800 which is housed with the ACME 360/50 and slaved to it.

Currently there are three general systems approaches.

1. Data logging by the computer and later data reduction by means of researcher-computer interacting programs. This has been documented in the Status Report of April 1 to September 30, 1966, Technical Report IRL 1054. In addition, a more complete technical report is being prepared at this time. This system employs some unique researcher-computer interaction concepts. Termed the BENDIX

system, it is currently being used on the LINC computer with the Bendix TOF and the Atlas CH-4.

2. A computer directed system (which we call Spectrum) in which the computer controls the mass spectrometer during data gathering phases. This system has been documented in Technical Report No. IRL-1062.

The computer directed systems realized to date have been: (1) LINC-EAI Quad-300 (2) LINC-Finnigan 1015, and (3) LINC-Bendix TOF.

3. A system capable of high mass resolution to link the ACME 360/50 with the AEI MS-9.

D. GLC/MS Computer Systems

The Finnigan Instruments Corporation model 1015 quadrupole mass spectrometer was installed and tested during January and February. A new computer interface, similar to that used with the EAI QUAD 300 and Bendix TOF was built and installed during this period. This system is functionally similar to that described in Technical Report IRL 1062, dated November 1, 1967.

It, however, has or makes provisions for the following improvements:

1. A useful computer controlled range to m/e 500, or possibly 750. This has included built-in provisions for 13 bit control resolution. (The present SPECTRUM uses only 12).
2. The FIC 1015 has a solid sample probe. Hence the mass spectrometer can analyze either solid samples or effluents of GLC.
3. It has an expanded control and sense multiplex system that allows up to a combination of 31 discrete controller sense functions. This is still further expandable.
4. It is adaptable to a variety of computers. The interface has a basic portion that needs only the simplest of digital inputs, a

12 or 13 bit word and 3 control lines. Almost any computer digital output configuration can be decoded to supply this. The input to the computer is a single analog line.

5. It has a unique nonlinear integrator.

The upper limit of integer resolution on the Finnigan instrument is said to be m/e=750. By using the highest m/e range of the 1015 (it has three) and the present SPECTRUM software, we were able to verify integer resolution at m/e-614 (10% valley definition). This was a first, although not the only, consideration in our decision to extend the system to at least m/e=500.

Measurements are now being taken to determine the suitability of a computerized operation of the Finnigan quadrupole over the greater range, m/e=500 or 750. For this greater range (with the same control voltage to the mass analyzer) a higher over all stability is required to allow unambiguous peak identification. To measure the peak more accurately, we will need the full 13 bit digital to analog converter capability to set the control voltage. The interface is currently being developed to the point where its stability will be primarily that of the digital to analog converter reference supply voltage (specified at 0.005% per degree F.). We still have to acquire accurate data on the stability of the quadrupole itself. Indications are that it is the instrument that will be the limiting factor. Presently the LINC with SPECTRUM limited to m/e=256 is being used. The reprogramming for the added range will be extensive. It has been decided to postpone this effort until ACME is able to provide instrumentation control. Despite the potential capability of ACME terminal connections, systems software is not yet available that can reasonably support a SPECTRUM-like system. At present, the LINC computer is quicker and more efficient. However, the LINC, since not time shared, cannot support all of the IRL systems that it can, one at a time, accommodate. Hence larger, time shared computers are attractive and a major effort is being made to tie into ACME. An alternative is more small computers, like

the LINC, each dedicated in a particular instrumentation problem, under ACME control.

Development of the extended mass range is being delayed until the time-shared ACME 360-50 computer as the control element can operate in the system. The 270Y input/output terminals for the IBM 360-50 or the 1800 could be used as an interface. Because of the apparent shorter development time, the connection to the 1800 will be made first. The organization of the software will be made compatible with either connection and applicable to either Bendix TOF or FIC 1015 mass spectrometer control.

The SPECTRUM software of the time-shared system will include an automatic calibration routine of the mass spectrometer. This function, not presently in the LINC system, will greatly reduce the set-up time presently required before an experiment is run.

The non-linear integrator effects a data compression somewhat analogous to a logarithmic amplifier in nonintegrating systems. As can be seen in Figure 7, the principle is to use additional integrating capacitors gated in by different zener diodes. This particular set of zener diodes and condensers allows full gain for signals of up to 1/3 maximum output value. Signals of larger value are subjected to more integrating capacitance and kept from saturation. It can be shown that the output signal retains a more nearly constant ratio of amplitude uncertainty (one bit in the a-to-d conversion) to actual signal level.

Precision components are not needed. Provision is made to allow the computer to determine the parameters of the more complex transformation. (This is by means of a control function to apply a calibrating current signal.) The computer program then applies an inverse transformation whenever a value of output voltage is logged.

Conventional linear integration may be diagramed as Figure 5.

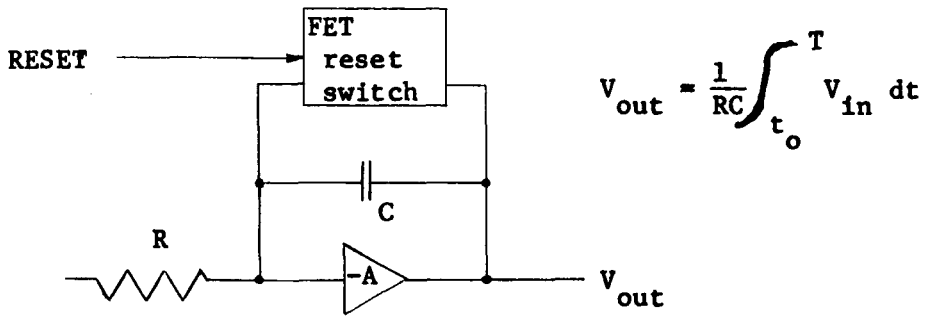

FIGURE 5

A conventional integration circuit.

For the case when V_{in} is kept constant and samples at various times, T, are recorded, V_{out} may be represented as in Figure 6.

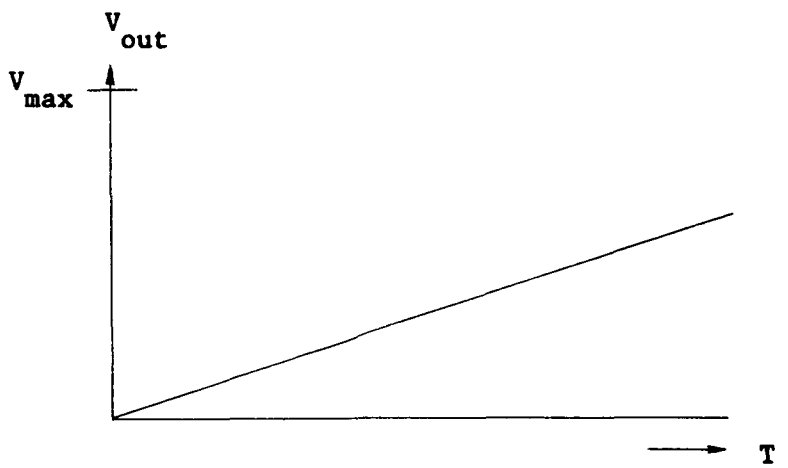

FIGURE 6

Typical output from a linear integrator.

The nonlinear integrator suitable for single valued inputs, shown

$V_{in} < 0$

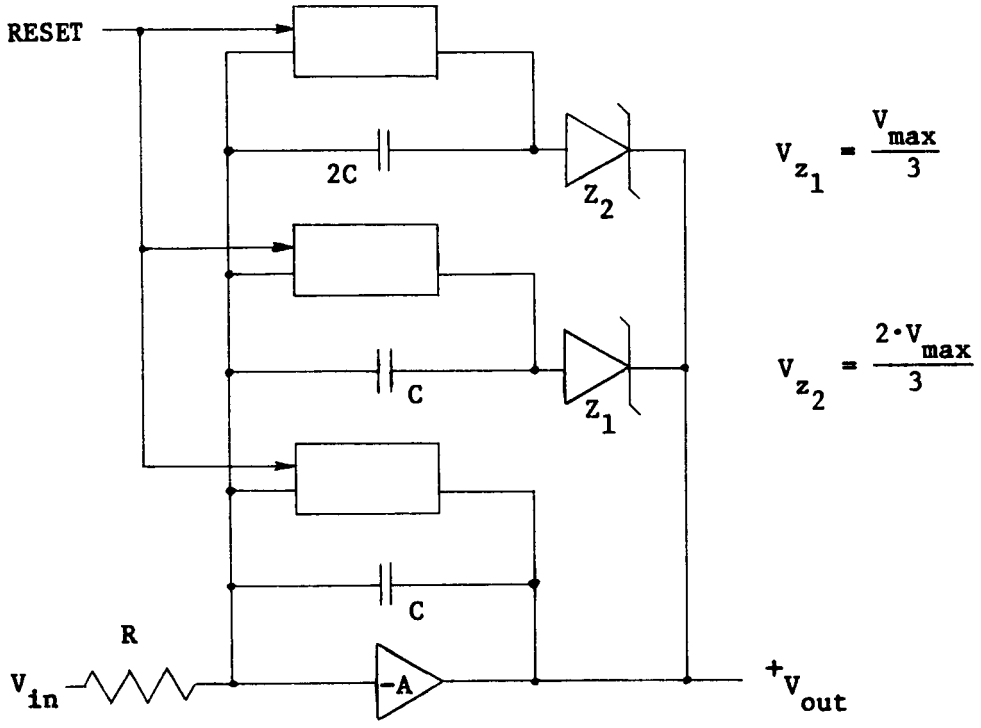

FIGURE 7

The circuit for the nonlinear integrator used.

Now the output for Figure 6 input conditions similar to those assumed but applied to the circuit shown in Figure 7 would be as shown in the final figure, 8.

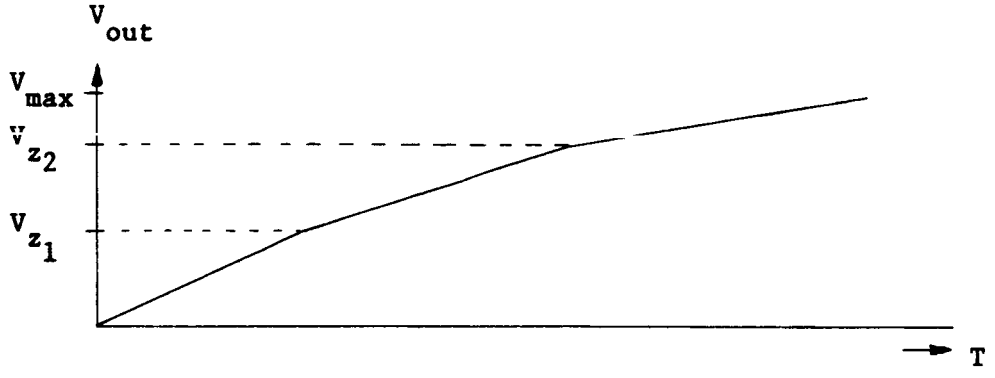

FIGURE 8

The output of the nonlinear integrator for a constant input signal.

E. Bendix TOF

No distinctly new data systems were applied to the Bendix TOF this reporting period. The BENDIX system was primarily used. However, the SPECTRUM system did prove quite workable and was distinctly advantageous in signal to noise characteristics. Its main drawback was lack of mass range. As noted elsewhere, the program logs only to m/e=256.

A paper, describing this development, was given at the Conference on Mass Spectrometry and Allied Topics at Pittsburgh, May 13 1968, and appended.

F. Atlas CH-4

The BENDIX system is now being applied to the Atlas CH-4. The output of the CH-4 mass spectrometer is normally sent through a logarithmic amplifier to a chart recorder. The amplifier has been modified to drive, through a coaxial cable, an analog input channel of the LINC. While the Atlas scans an m/e range of about 30 to 500 (over a period of several minutes) the LINC samples the data using a program developed for the Bendix TOF mass spectrometer. Several such runs have been made. The data has been processed and presented as a bar graph of amplitude versus mass, via a Calcomp plotter. After preliminary investigation using the existing software on the LINC, we plan to develop a more suitable system utilizing the ACME 360/50 together with the IBM model 1800 computer.

G. MS-9

The hardware for the high resolution AEI MS-9 mass spectrometer connection to the ACME 360/50 was completed during the reporting period. This connection is to be made via the 270X-Y. Systems support for the

270X-Y and the difficulties described elsewhere in this report have prevented completion of the system connection. Enough of it has been run either in small sample runs, or slow rates to nearly completely check out the hardware, but not enough support has been given to take data from the mass spectrometer.

A detailed description of this system will be given in a subsequent report.

The approach is not a particularly new one, it has been generally accomplished by a number of laboratories. The hardware should not only do its design task, but have a great deal of extra capability that will allow it to be the basis of computer control and data processing for a number of other mass spectrometers and instruments.

It consists of a rack of equipment including power supplies, timing oscillators, signal conditions, and one of the manually switchable 270Y's. Attached are the magnet controls and electrometer. Central to this equipment is the digital control and A-to-D converter. It is expected that real time data acquisition will begin shortly.

H. Computer Manipulation of Chemical Hypotheses

The generalization of DENDRAL to ring systems has been completed and will be embodied in a technical report to be released shortly.[4] The attached table of computer output illustrates the successful manipulation of positional isomerisms.

The input problem is to produce all the isomers of $C_9H_{12}O$ containing a benzene ring.

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[4] Joshua Lederberg, "DENDRAL, A System for Computer Construction, Enumeration and Notation of Organic Molecules as Tree Structures and Cyclic Graphs", Part III, Complete Chemical Graphs; Embedding Rings in Trees

```
(ISOMERS (QUOTE C9H120))
(APRIL-14-1968 VERSION)
C3O*PHEN*H12
(RING *PHEN* COMPOSITION ((U . 4.) (C . 6.)) VALENCE (1. 1. 1. 1. 1.
1.) SYMMETRIES ((2. 3. 4. 5. 6. 1.) (3. 4. 5. 6. 1. 2.) (4. 5. 6. 1. 2
. 3.) (5. 6. 1. 2. 3. 4.) (6. 1. 2. 3. 4. 5.) (6. 5. 4. 3. 2. 1.) (5.
4. 3. 2. 1. 6.) (4. 3. 2. 1. 6. 5.) (3. 2. 1. 6. 5. 4.) (2. 1. 6. 5. 4
. 3.) (1. 6. 5. 4. 3. 2.)) UNIQUE-NODES (1.))
MOLECULES       NO DOUBLE BOND EQUIVS
  1.    CH2..  C2H5   O.(*PHEN* 1.)H5 ,
  2.    CH2..  C2H5   (*PHEN* 1. 2.)H4.OH ,
  3.    CH2..  C2H5   (*PHEN* 1. 3.)H4.OH ,
  4.    CH2..  C2H5   (*PHEN* 1. 4.)H4.OH ,
  5.    CH2..  CH2.OH   CH2.(*PHEN* 1.)H5 ,
  6.    CH2..  CH2.OH   (*PHEN* 1. 2.)H4.CH3 ,
  7.    CH2..  CH2.OH   (*PHEN* 1. 3.)H4.CH3 ,
  8.    CH2..  CH2.OH   (*PHEN* 1. 4.)H4.CH3 ,
  9.    CH2..  O.CH3   CH2.(*PHEN* 1.)H5 ,
 10.    CH2..  O.CH3   (*PHEN* 1. 2.)H4.CH3 ,
 11.    CH2..  O.CH3   (*PHEN* 1. 3.)H4.CH3 ,
 12.    CH2..  O.CH3   (*PHEN* 1. 4.)H4.CH3 ,
 13.    O..  C2H5   CH2.(*PHEN* 1.)H5 ,
 14.    O..  C2H5   (*PHEN* 1. 2.)H4.CH3 ,
 15.    O..  C2H5   (*PHEN* 1. 3.)H4.CH3 ,
 16.    O..  C2H5   (*PHEN* 1. 4.)H4.CH3 ,
 17.    (*PHEN* 1. 2.)H4..  C2H5   CH2.OH ,
 18.    (*PHEN* 1. 3.)H4..  C2H5   CH2.OH ,
 19.    (*PHEN* 1. 4.)H4..  C2H5   CH2.OH ,
 20.    (*PHEN* 1. 2.)H4..  C2H5   O.CH3 ,
 21.    (*PHEN* 1. 3.)H4..  C2H5   O.CH3 ,
 22.    (*PHEN* 1. 4.)H4..  C2H5   O.CH3 ,
 23.    CH...  CH3   CH3   O.(*PHEN* 1.)H5 ,
 24.    CH...  CH3   CH3   (*PHEN* 1. 2.)H4.OH ,
 25.    CH...  CH3   CH3   (*PHEN* 1. 3.)H4.OH ,
 26.    CH...  CH3   CH3   (*PHEN* 1. 4.)H4.OH ,
 27.    CH...  CH3   OH   CH2.(*PHEN* 1.)H5 ,
 28.    CH...  CH3   OH   (*PHEN* 1. 2.)H4.CH3 ,
 29.    CH...  CH3   OH   (*PHEN* 1. 3.)H4.CH3 ,
 30.    CH...  CH3   OH   (*PHEN* 1. 4.)H4.CH3 ,
 31.    CH...  CH3   (*PHEN* 1.)H5   CH2.OH ,
 32.    CH...  CH3   (*PHEN* 1.)H5   O.CH3 ,
 33.    CH...  OH   (*PHEN* 1.)H5   C2H5 ,
 34.    (*PHEN* 1. 2. 3.)H3...  CH3   CH3   CH2.OH ,
 35.    (*PHEN* 1. 2. 4.)H3...  CH3   CH3   CH2.OH ,
 36.    (*PHEN* 1. 3. 2.)H3...  CH3   CH3   CH2.OH ,
 37.    (*PHEN* 1. 3. 4.)H3...  CH3   CH3   CH2.OH ,
 38.    (*PHEN* 1. 3. 5.)H3...  CH3   CH3   CH2.OH ,
 39.    (*PHEN* 1. 4. 2.)H3...  CH3   CH3   CH2.OH ,
 40.    (*PHEN* 1. 2. 3.)H3...  CH3   CH3   O.CH3 ,
 41.    (*PHEN* 1. 2. 4.)H3...  CH3   CH3   O.CH3 ,
 42.    (*PHEN* 1. 3. 2.)H3...  CH3   CH3   O.CH3 ,
 43.    (*PHEN* 1. 3. 4.)H3...  CH3   CH3   O.CH3 ,
 44.    (*PHEN* 1. 3. 5.)H3...  CH3   CH3   O.CH3 ,
 45.    (*PHEN* 1. 4. 2.)H3...  CH3   CH3   O.CH3 ,
 46.    (*PHEN* 1. 2. 3.)H3...  CH3   OH   C2H5 ,
 47.    (*PHEN* 1. 2. 4.)H3...  CH3   OH   C2H5 ,
 48.    (*PHEN* 1. 2. 5.)H3...  CH3   OH   C2H5 ,
 49.    (*PHEN* 1. 2. 6.)H3...  CH3   OH   C2H5 ,
 50.    (*PHEN* 1. 3. 2.)H3...  CH3   OH   C2H5 ,
 51.    (*PHEN* 1. 3. 4.)H3...  CH3   OH   C2H5 ,
 52.    (*PHEN* 1. 3. 5.)H3...  CH3   OH   C2H5 ,
 53.    (*PHEN* 1. 3. 6.)H3...  CH3   OH   C2H5 ,
 54.    (*PHEN* 1. 4. 2.)H3...  CH3   OH   C2H5 ,
 55.    (*PHEN* 1. 4. 3.)H3...  CH3   OH   C2H5 ,
 56.    C....  CH3   CH3   OH   (*PHEN* 1.)H5 ,
 57.    (*PHEN* 1. 2. 3. 4.)H2....  CH3   CH3   CH3   OH ,
 58.    (*PHEN* 1. 2. 3. 5.)H2....  CH3   CH3   CH3   OH ,
 59.    (*PHEN* 1. 2. 4. 3.)H2....  CH3   CH3   CH3   OH ,
 60.    (*PHEN* 1. 2. 4. 5.)H2....  CH3   CH3   CH3   OH ,
 61.    (*PHEN* 1. 2. 4. 6.)H2....  CH3   CH3   CH3   OH ,
 62.    (*PHEN* 1. 3. 5. 2.)H2....  CH3   CH3   CH3   OH ,

DONE
```

IV. Cell Separation

A. Introduction

The work on cell separation has proceeded to the point where there are now three instruments capable of carrying out worthwhile biological experiments. The first of these is a volumetric cell separator, the second is a high speed fluorescent cell separator and the third is a cell separator and identifier developed by the Watson Labs of IBM under the direction of Dr. L. A. Kamentsky. While our ultimate goal is the possible application of these principles to the biological exploration of the planets, our present efforts are directed toward current biological and medical problems. This has made it possible for us to gain the active participation of other scientists here at the Medical School working under different research grants. These have included Professor H. S. Kaplan, Executive Head of the Radiology and Radiotherapy, and Dr. I. L. Weissman, Department of Radiology, American Cancer Society, Grant E-439 and NIH grants CA3352-11, CA05008-11 and CA0583807; Professor George Hahn, Department of Radiology, NIH Research Grant CA4542-09; Professor Leonard Herzenberg, Department of Genetics, NIH Research Grant CA-04681; Professor R. Kallman, Department of Radiology, NIH Research Grant CA3353-11.

B. Present Biological Program

Initially, we have established criteria for cell types which could be used as assay material while developing the prototype instruments leading to uses of instruments designed for cell separation in experiments that are biologically significant. These criteria are:

1. Cells of at least two major size categories.
2. Cells which are not adherent to one another and which can be maintained in single cell suspension.

3. Cells which can be reproducibly obtained and maintained with good viability.
4. Cells which can be divided into subclasses according to biological function, subclasses which might be related to cell volume or cell fluorescence.

For these reasons, we chose to work on thoracic duct populations of lymphocytes, which are:

1. Divided into at least 3 size subclasses.
2. Naturally obtained in single cell suspension and do not form clumps of 2 cells or greater.
3. Can be maintained in a viable state with appropriate media for as long as 24 hours.
4. Have at least two significant biological criteria for size-subclass separation:
   a. DNA synthesis: Large and medium thoracic duct lymphocytes are in a continuous cycle of cell division. At least 80% of their cell cycle is devoted to DNA synthesis, and therefore short-term incubation of thoracic duct lymphocyte populations with radioactive DNA precursors will selectively label large and medium cells. Small lymphocytes only rarely divide.
   b. Small lymphocytes are "immunologically competent cells", whereas populations of large and medium lymphocytes are not. We have developed rapid and easy methods for testing immunological competence.
   c. Both of the above assays detect these biological functions in viable cells only. Therefore, since we have a system in which only viable cells fluoresce, we can test the fluorescent cell separator's action also.

The following are a few of the medically and biologically significant experiements we hope to perform:

1. Test purified cell populations for cell-cell interactions in the induction, development, and execution of the immune response (in conjunction with Professor Herzenberg and Dr. Weissman).
   a. Isolation of antigen-processing cells by phagocytosis of fluorogenic substrate.
   b. Isolation of immunologically competent cells by size.
   c. Isolation of antibody-forming cells by size following adherence of large antigenic particles.
2. Isolation of cells in mitosis by size criteria in order to establish cell lines in vitro which are synchronously cycling. These will be useful to determine the actual cellular and molecular events which determine the differential sensitivity of cells to radiation and certain drugs as a function of their place in the cell cycle (in conjunction with Professor George Hahn, Department of Radiology).
3. Detection and isolation of cancer cells in the blood stream (metastases) in order to determine the type of cancer therapy most appropriate for the patient (in conjunction with Professor H. S. Kaplan, Executive Head of Radiology and Radiotherapy and Professor R. Kallman, Radiology Department).
4. Isolation and testing of cell types in Hodgkins Disease, (a cancer of the lymph node system) in order to determine:
   a. the malignant cell, its biochemistry and radiosensitivity.
   b. the cell type (in these patients) responsible for widespread immunological deficiency, and how this deficiency is maintained (in conjunction with Professor H. S. Kaplan).
5. Isolation and testing of the cell type in the bone marrow theoretically designated as the "stem" cell, which is responsible for redevelopment of normal blood cell types following irradiation (in conjunction with Dr. Weissman). (NASA and the Air Force already have supported projects and symposia aimed at answering just this question, for the purpose of possible treatment of radiation exposure in space.)

C. Volumetric Cell Separator

We are continuing the development of the volumetric cell separator reported in the previous reports of this laboratory, IRL-1056 and IRL 1061.

As reported then the clogging of the orifice structure was much too frequent for any practical applications of the apparatus. After extensive investigation of this problem, some modifications were incorporated in the fluid intake system of the apparatus so that the clogging has been practically eliminated. The solutions must be carefully handled to assure that build-up of large foreign matter within the orifice does not occur and eventually will clog the apparatus. In recent experiments we were able to run the apparatus for several days, several hours each day, without clogging. Besides the modifications in the fluid intake system, the orifice structure was also modified for ease of assembly and manufacturing. Figure 9 shows the orifice structure. Note that the alignment problem of the double aperture structure has been eliminated by virtue of the manufacturing process of the two piece structure. In Figure 10 the entire mechanical apparatus is shown. The liquid is inserted at opening A. Air is introduced at point B. The air is filtered by a sintered glass filter incorporated in the glass reservoir C. The liquid is then fed through the tube D into the filter housing E. This filter housing incorporates a 20 micron nickel filter. The solution then is fed into the head of the assembly which incorporates the ultrasonic generator F and the orifice structure G. The liquid under pressure emerges as a stream at point H. This unstable liquid jet is then passed through the charging-deflecting and collecting assembly shown in Figure 11. The charging ring A applies a charge that is proportional to the volume of the particle detected at the orifice just before the stream breaks into uniform droplets. The deflection plates B form an electrical field deflecting the charged droplets proportionally to their charge. The collecting

assembly C contains an adjustable member with six cavities spaced about .050 inches apart. In turn the liquid collected in these cavities is collected by means of flexible tubing into large containers with the aid of a vacuum.

Preliminary results with the apparatus indicate that detection of small particles can be achieved and separation by deflection can be attained.

Following is a theoretical analysis of the resistivity changes expected and a correlation with some preliminary results.

The insulating orifice, as has been reported, is composed of a glass orifice having an aperture of d = 80 micron diameter with a length of ℓ = 100 micron. The resistance of the sylinder is

$$R = C \frac{L}{A} = C \frac{4\ell\rho}{\pi d^2}$$

If an insulating particle of diameter d_1 and length ℓ_1 enter the aperture, the resistance will be

$$R_1 = C \frac{4(\ell-\ell_1)}{\pi d^2} + \frac{4\ell_1}{\pi(d^2-d_1^2)}$$

therefore

$$\Delta R = R_1 - R \simeq C \frac{4\ell_1}{\pi d^2}\left(\frac{d_1}{d}\right)^2$$

where C is the resistivity of the conductive suspending solution. For normal saline solution:

$$C \simeq 50 \text{ ohm-cm}$$

Therefore for the dimensions given above:

$$R = 50 \times \frac{4 \times 100 \times 10^{-4}}{\pi(80 \times 10^{-4})^2} = 10{,}000 \text{ ohm.}$$

The experimental measured resistance of the orifice is 11,500 ohms.

For a particle having the dimensions of human red blood cell (d ≃ 6 microns and $\ell_1 \simeq 2$ micron):

$$\Delta R = 50 \frac{4 \times 2\ 10^{-4}}{\pi\ (80 \times 10^{-4})^2} \left(\frac{6}{80}\right)^2 = 1.12 \text{ ohms}$$

With a detection current of 380×10^{-6} amperes flowing through the orifice the voltage differential due to this size cell in the orifice will be:

$$\Delta V = 1.12 \times 380 \times 10^{-6} = 425 \times 10^{-6} \text{ volts.}$$

In our apparatus this voltage is amplified by 10^3. Therefore the output amplified voltage should be:

$$\Delta V_o = 425 \times 10^{-6} \times 10^3 = .425 \text{ volts.}$$

Figures 12 and 13 show the signal derived by passing human red blood cells through the cell detector with the experimental conditions indicated above. The vertical sensitivity of both pictures is 0.200 volts/cm. Figure 12 was taken with a horizontal sensitivity of 1 msec/cm while Figure 10 with a sensitivity of 50 μ sec/cm. It is obvious from

Figure 13 that the concentration of the solution was too great since coincidence is observed.

The noise in the system is less than 20 mv which is equivalent to:

$$V_{noise} = 2- \times 10^{-3} \times 10^{-3} = 20 \times 10^{-6} \text{ volts}$$

at the input.

This represents an equivalent noise resistance of

$$R_{noise} = \frac{2 \times 10^{-6}}{38 \times 10^{-6}} \simeq .052 \text{ ohms.}$$

This noise resistance can be further correlated to the equivalent particle volume,

$$R_{noise/unit\ \ volume} = 9.3 \times 10^{-4} \text{ ohms}/\mu^3$$

or a noise level equivalent to a $2.6\mu^3$ particle. All of the above theoretical results correlate very closely to the experimental signals observed in the apparatus. Some preliminary experimentation with white cells was performed, with somewhat disturbing results. First indications are that the apparatus tends to destroy the cells. This phenomenon has not been investigated extensively, but it is believed that the rapid changes in pressure and the additional vibration induced by the ultrasonic generator causes these cells to burst. Further investigation on the viability of white cells is scheduled in the near future. Our future plans include separation of a blood cell mixture, white from red.

D. <u>High Speed Fluorescent Cell Separation</u>

The cell separation system described in previous reports has been modified mechanically, optically and electronically for the specific

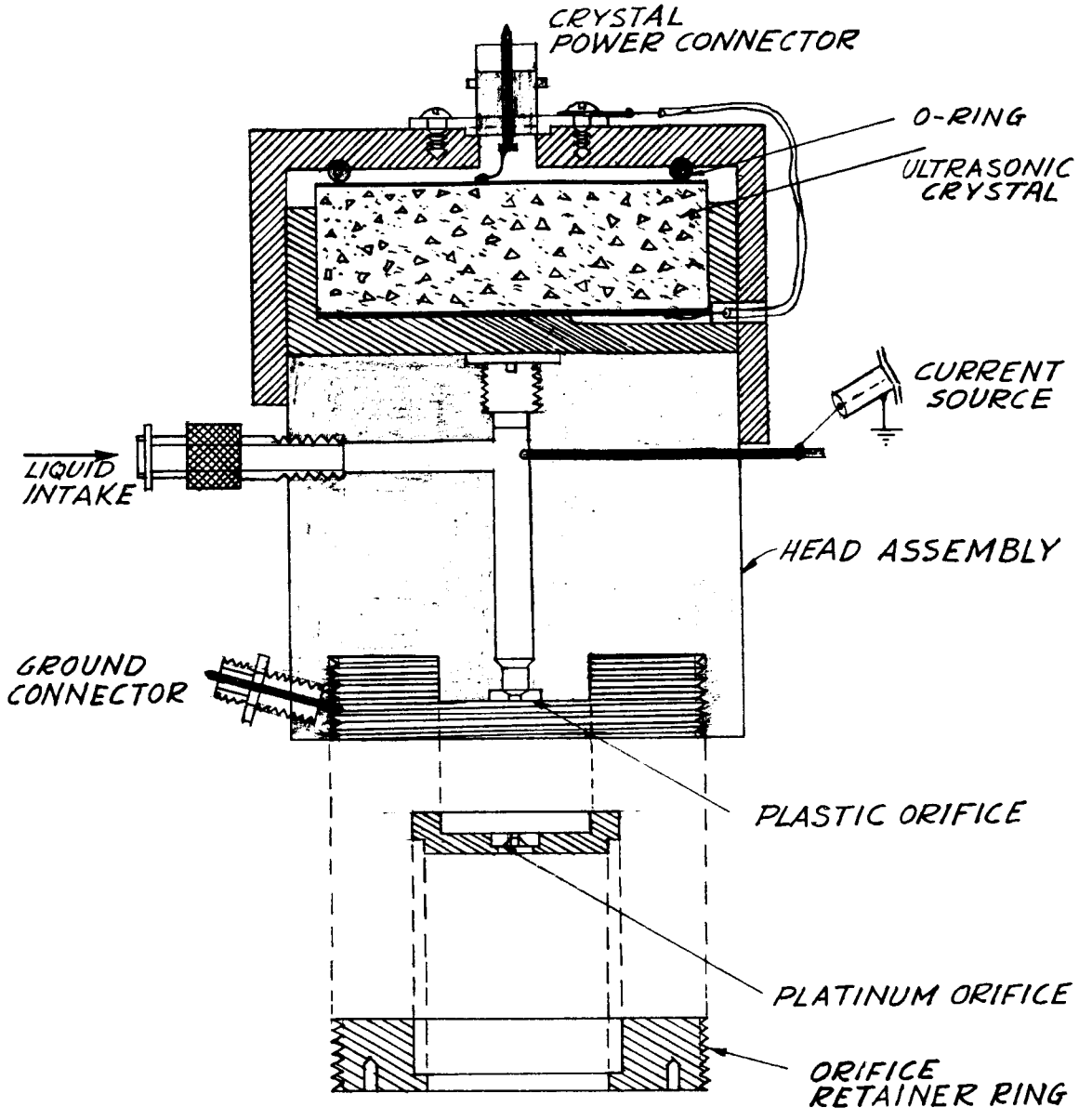

FIGURE 9
SEPARATOR ASSEMBLY

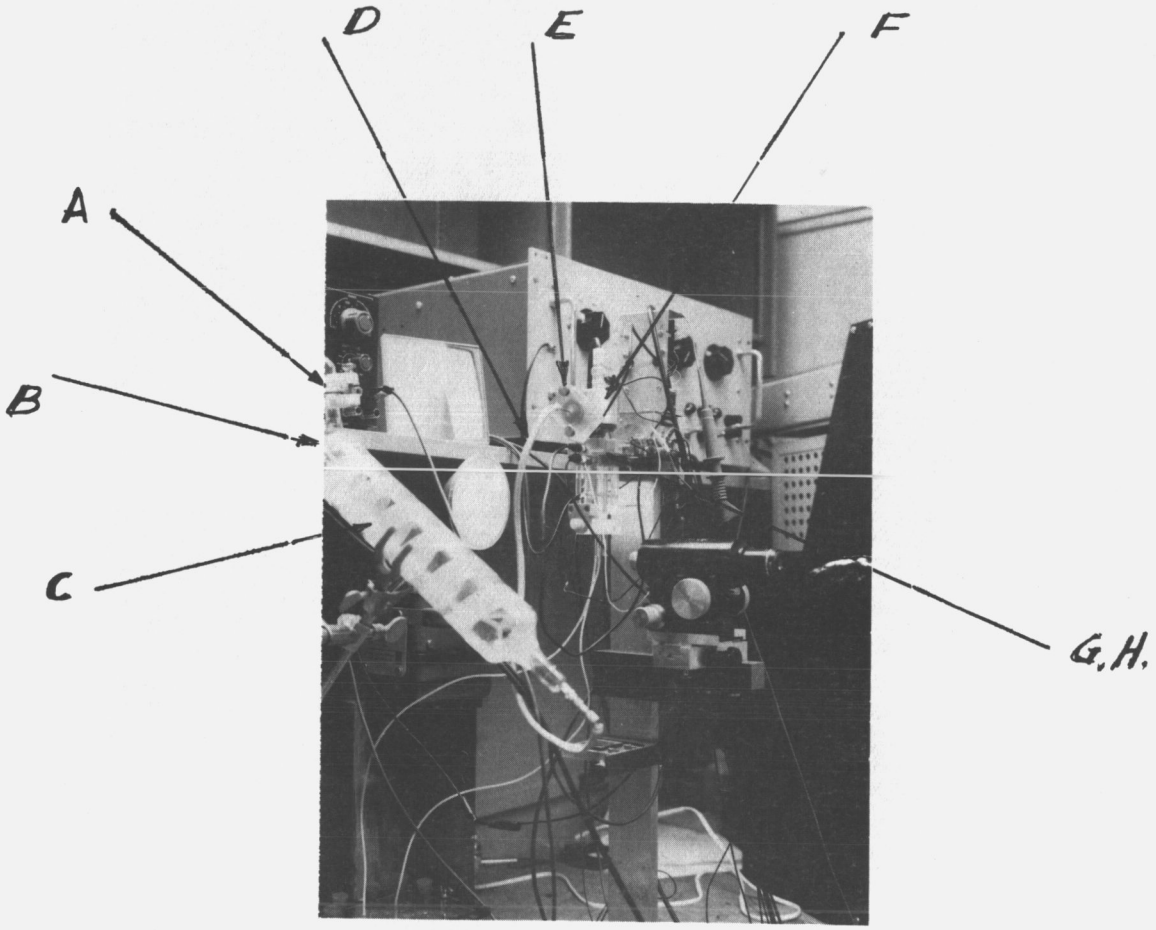

FIGURE 10

CELL SEPARATOR

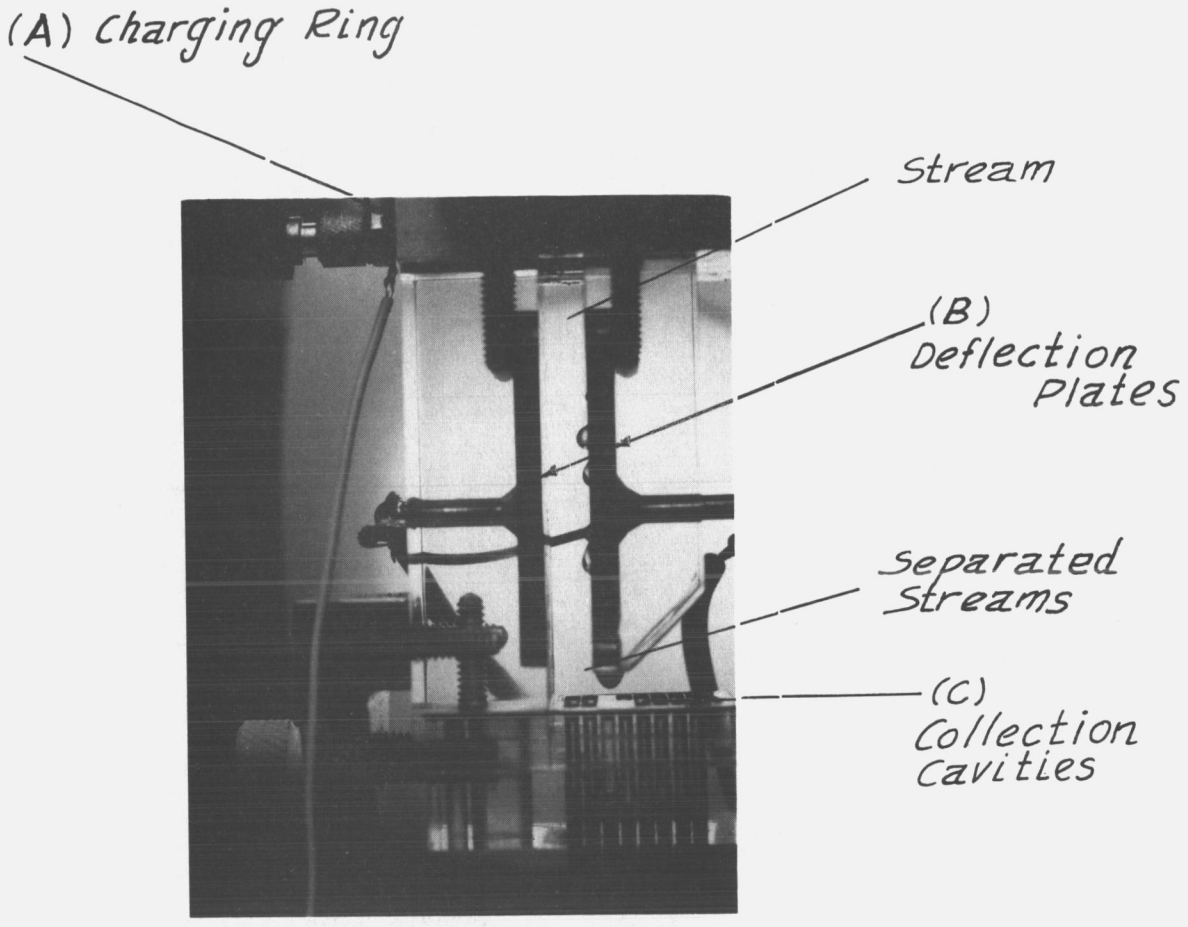

FIGURE 11

Charging, Deflecting, Collecting Apparatus.

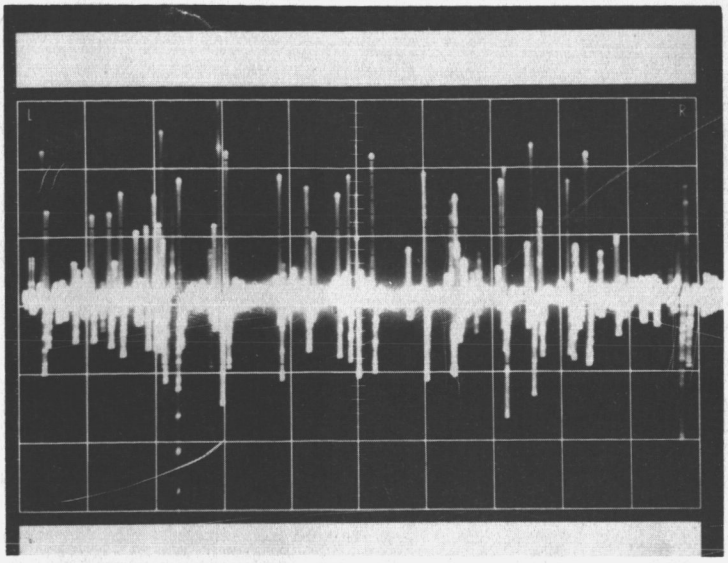

FIGURE 12

200 mv/cm vertical
1 msec/cm horizontal

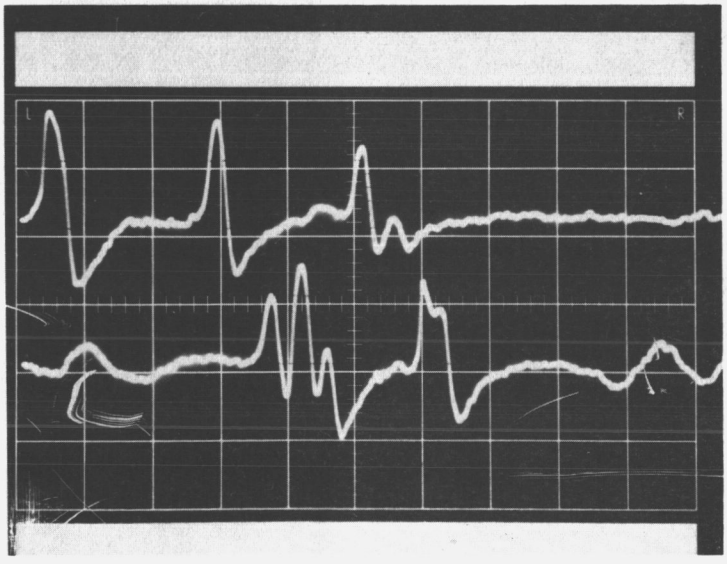

FIGURE 13

200 mv/cm vertical
500 μsec/cm horizontal

purpose of routine separation of fluorescent cells from a heterogenous sample. All major functions of the system have performed satisfactorily.

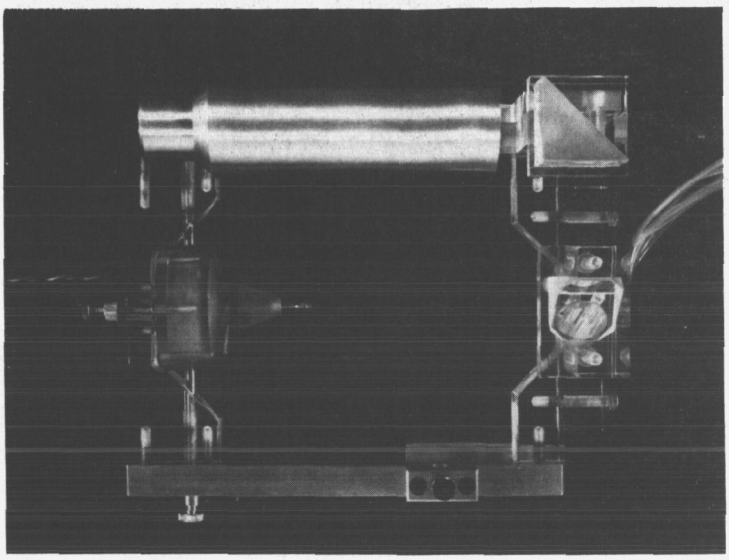

FIGURE 14

Figure 14 shows the present form of the jet forming and deflection module. The transducer mounting has been fitted with X, Y and Z positioning adjustments for precise initial aiming of the droplet stream. A secondary low magnification optical train facilitates observation of the droplets upon arrival at a multiple port collector. Each port of the collector, shown in Figure 15, directs separated droplets and cells to individual 15 ml. tubes for subsequent tests. A low pressure vacuum common to all tubes and ports prevents liquid spillage by removing accumulated liquid. The tube holder is shown in Figure 16.

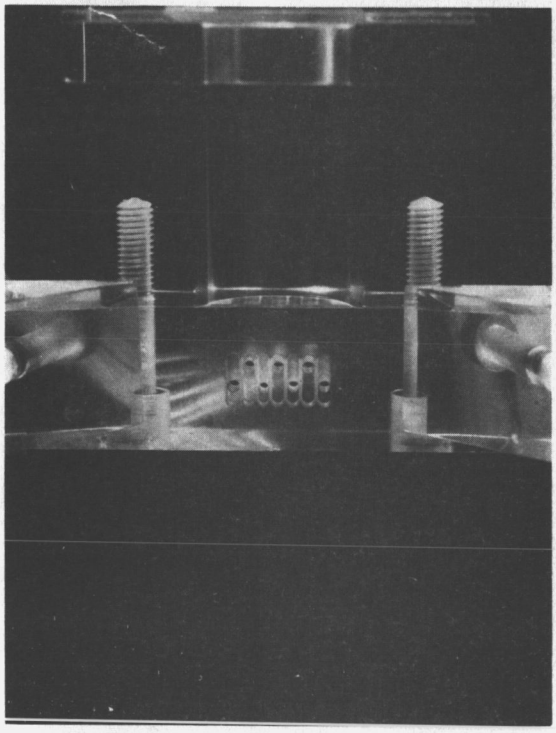

FIGURE 15

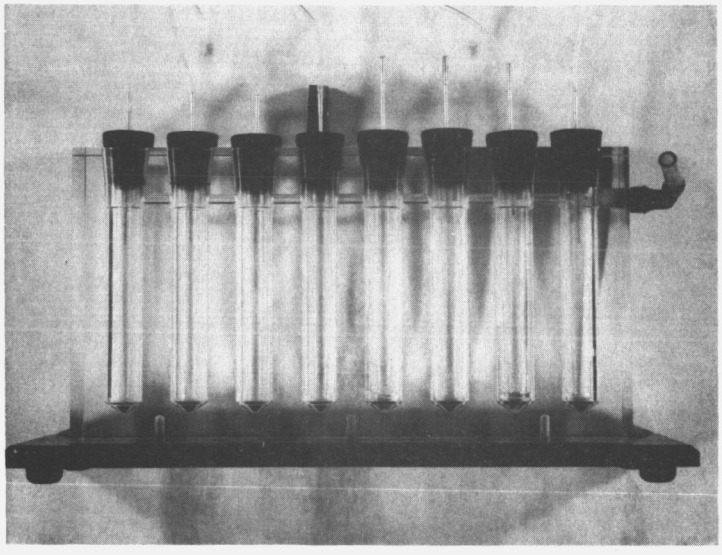

FIGURE 16

A test waveform consisting of a 25 μsecond + 50 volt pulse applied every fifth droplet period produced the deflection shown In Figure 17.

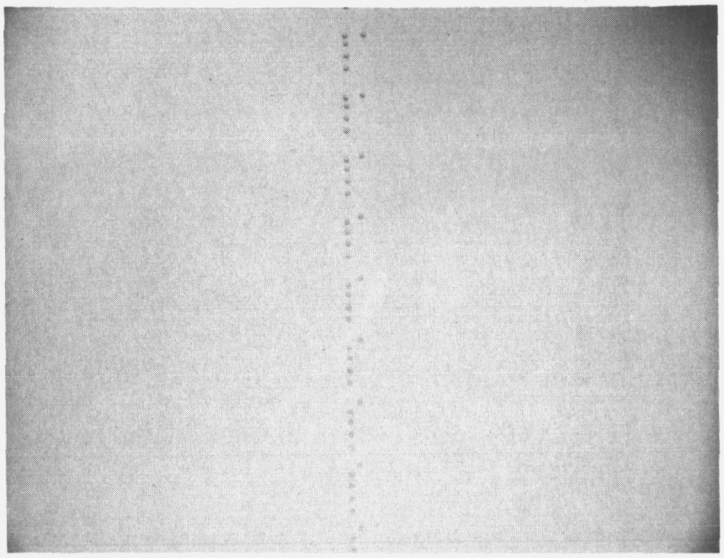

FIGURE 17

In this region of the droplet stream the droplets are passing between the deflection plates and an increasing deflection of the charged droplet is noted. Figure 18 shows the result of applying a linear ramp of + 125 volts and 5 droplet periods duration to charge the stream.

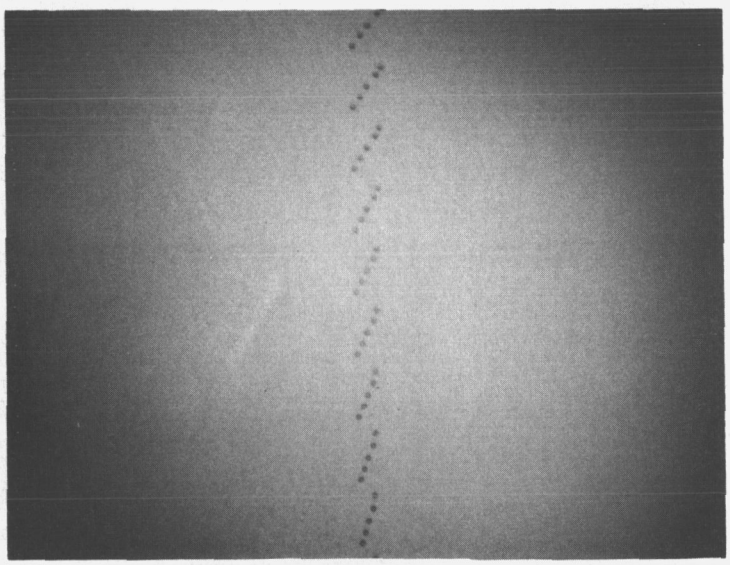

FIGURE 18

In the instances noted, the droplet deflections were produced by charging pulses synchronously related to the droplet generation rate. Because fluorescent cells will appear randomly spaced in the jet stream an alternative method of droplet charging will be used. The unbroken jet stream will be so positioned on the microscope stage that a point in the jet stream some 150 microns from the forming droplet will be optically centered. A circular mask in the projection plane of the microscope will permit a multiplier phototube to receive fluorescence information from a 100 micron diameter area centered on the point noted. When a fluorescent cell is detected, a pulse delay will be initiated lasting for some 12.5 microseconds. At the end of this delay the unbroken jet stream will be charged for a period slightly in excess of a droplet formation period. The delay pulse, the early start, and late end of the charging pulse will increase the probability of the target cell being within the charged droplet at the time the droplet separates. The droplet so charged will be guided to a defined collection port. Some uncertainties evidently remain. A nonfluorescent cell could pass undetected into the forming droplet immediately before or after a fluorescent cell. A careful choice of cell suspension density would reduce this possibility to the desired degree. Both the delay and charging pulses can be adjusted in length to optimize separation.

Several tests have been made of the instrument. Detection sensitivity and deflection accuracy are satisfactory. Total and partial blockages of the jet nozzle have occurred but not often enough to constitute a serious problem. It would nevertheless be of some value to continuously monitor an experiment. At the present time the optical information reaches either a phototube or the eye of the experimenter. Work will be carried out to permit simultaneous illumination of the droplet stream by both arc and stroboscopic light sources. A low loss beam splitter with suitable color filter would then relay morphological and positional information to the experimenter for possible remedial action.

E. IBM Cell Separator

1. Instrumentation and Methods

We have been loaned a cell separator and identifier developed by the Watson Laboratories of IBM under the direction of Dr. L. A. Kamentsky[5]. This unit operates on photoelectric signals generated by passage of cells through an illuminated channel (100μ cross section in our experiments). As many as four different spectral regions can be viewed simultaneously by four separate photomultiplier tubes, through the use of appropriate filters and/or dichroic mirrors.

Our initial experiments with this unit have been directed toward studies of fluorochromasia, the appearance of fluorescence in cells because of enzymatic hydrolysis of nonfluorescent fluorescein diacetate (FDA) to give fluorescein, and its retention within the cell[6]. We are attempting to learn if this phenomenon can be used as a measure of viability. Rat thoracic duct lymphocytes have been used in most of the work, furnished by Dr. Thomas Barclay of the Stanford Radiology Department. In addition we have run some tests on chicken and human blood plasma.

In these tests fluorochromasia is developed by mixing the samples with 2 μ liters per ml. of a .5% solution of FDA in acetone. (In some cases interfering substances of low molecular weight are removed by spinning the cells down in a low speed centrifuge and resuspending in the chosen medium prior to adding FDA.) The resulting mixture is diluted to a concentration of about 1.5×10^5 cells/ml before passing through the channel. The channel is illuminated with blue light from a high pressure mercury source, using a 4 mm BG 12 filter and a dark field condenser.

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[5] L.A. Kamentsky, M. R. Melamed and H. Derman, Science 150, 630, 1965.

[6] B. Rotman and B. W. Papermaster, Proc Natl Acad Sci, 55, 134, 1966.

A photomuliplier is used to detect the particles which scatter the incident light. The number of such particles is determined with a Beckman model 7360 events per unit time meter and is assumed to represent total cell count. The number of fluorescent cells is counted similarly after the emergent light is passed through a 1 mm GG14 filter which will transmit the fluorescent signals but not the incident light. Both counts are relative, since signals are not always high with respect to the background noise. However, comparisons of scatter counts with hemocytometer counts on the same sample indicate that essentially all cells give detectable signals in this channel. Noise in the fluorescence channel is governed by fluorescence of fluorescein (from hydrolyzed FDA) in the liquid medium, and thus varies from time to time and from sample to sample. To minimize problems from this variation most counts in this channel have been made at a trigger level corresponding to four times the background noise level without fluorescent liquid in the channel. In an attempt to remove this problem, we are currently trying to construct channels approximately 25μ in cross sections. Such small channels have been found by Dr. Kamentsky to reduce the noise dramatically in his experiments, as might be expected.

An X-Y oscilloscope is available on the instrument, with shaping circuits allowing it to present a dot display of the peak voltage generated by each pulse.

2. Results

First experiments were intended to determine general characteristics of the fluorachromatic reaction.

a. Correlation of scatter and fluorescence
Typical photographs from the oscilloscope are presented in Figures 19 and 20, taken during assay of a cell suspension in phosphate buffered saline (PBS). In these figures

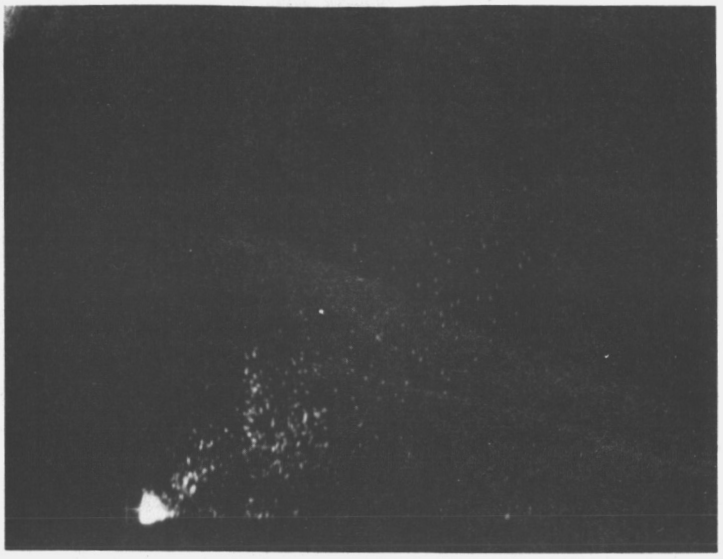


FIGURE 19

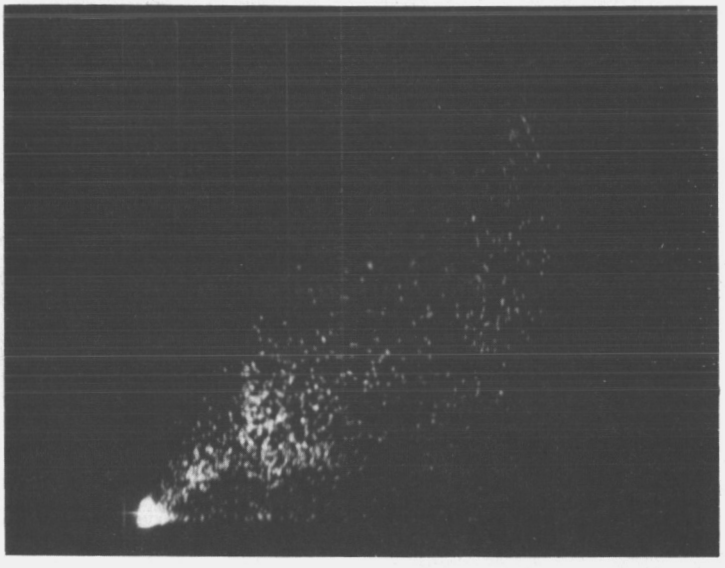


FIGURE 20

the horizontal axis represents scatter, the vertical axis fluorescence. No well defined grouping of the dot pattern has been apparent on any of the photographs taken, indicating little correlation between the two signals.

b. Ability to restain.

The fluorochromatic reaction requires that cells split FDA, but retain the resulting fluorescein for some period of time (generally hours). The fluorescein appears to be released into the suspending medium eventually, and the number of fluorescent cells decreases to zero. The ability of cells to split FDA again after such decay was checked by spinning down such cells and retreating with FDA. No appreciable loss in staining ability was found, indicating that the cells were probably still viable.

c. Effect of KCN.

Bodmer[7] has found that KCN and other metabolic inhibitors retard loss of fluorochromasia. We corroborated this finding but found that concentrations able to exert appreciable effects prevented restaining of the type referred to in b above and thus probably led to the death of the cells. Such an effect of a metabolic inhibitor indicates that transport of the fluorescein across the cell membrane is an active process, utilizing metabolic energy.

d. Effect of medium on fluorochromasia.

A series of tests was made using various media to determine the effect of the media on the fluorochromatic reaction. Media used included phosphate buffered saline, Ringers solution, lactated Ringers solution, 199 growth medium, and combinations of each of these with 1 to 10% fetal calf serum. The most effective medium for maintaining fluorochromasia was medium 199 with 5 to 10% fetal calf serum added.

Development of Fluorochromasia was relatively slow, requiring

[7] W. Bodmer, Genetics Department, Stanford University, Personal Communication.

from 15 minutes to an hour to reach peak value. Decay at high concentration required several hours to drop to 50% of peak, but varied widely depending on cell treatment. Decay at lower concentrations was more rapid.

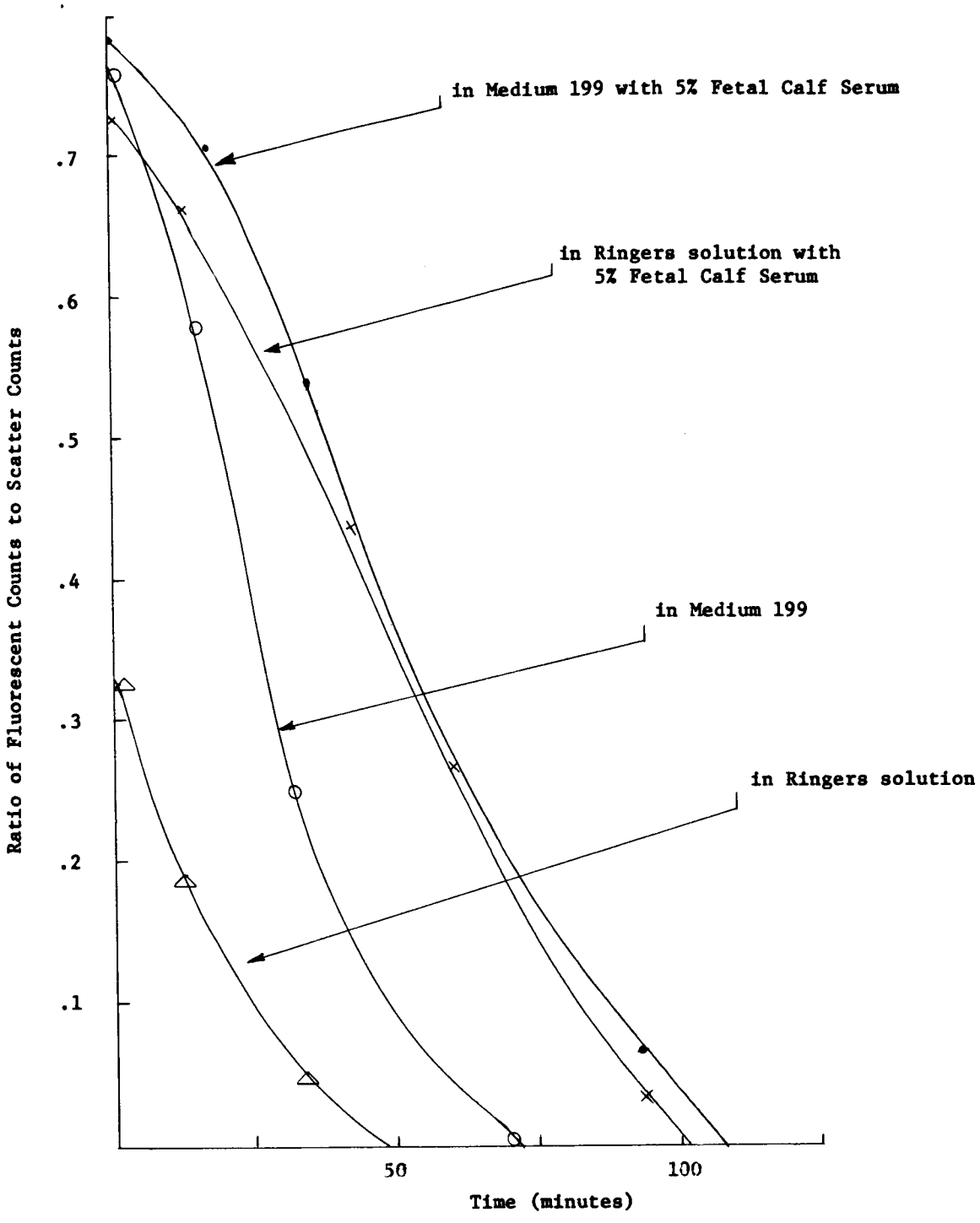
Decay of fluorescence was slowest in the fetal calf serum (FCS) solutions. This may indicate increased cell viability in these solutions. There may also have been physical adsorption of the FCS proteins to the cell walls, providing an extra barrier to release of the polar fluorescein molecules.

The decay was more rapid in dilute solutions. However, the variation with concentration was less than would have been expected if passive diffusion governed, again indicating a probability of active transport of fluorescein. The effect of dilution was greatest late in the experiment, probably because of loss of viability of the cells as time went on.

Data for experiments comparing the maximum FC developed in various solvents as a function of cell age since collection are shown in Figure 21. In all cases the cells were kept in the original solution until just before the measurements. It is obvious from these data that the media containing fetal calf serum were able to maintain the fluorochromasia of cells longer.

e. Effect of machine treatment on fluorochromatic ability.
A series of experiments were conducted to determine the effect of passage through this instrument, or through such other apparatus as the Coulter cell counter or the cell separation units being developed here, on the ability to develop FC. Initial tests using PBS as the suspension medium indicated that only the present equipment maintained this ability.

However, use of various solutions of fetal calf serum in PBS improved the situation strikingly, with maximum retention in 5 to 10% fetal calf serum. Since such concentrations also maintain FC in aged cell populations, the cells passed through the various instruments may still have been damaged to some extent. Conventional viability tests to determine this, as well as the relationship between FC ability to grow, are now in progress.



Fluorochromasia as a function of storage time and suspending medium.

FIGURE 21

PUBLICATIONS

PAPERS AND REPORTS

October 1, 1967 to March 31, 1968

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1. R. B. Tucker, "A Mass Spectrometer Data Acquisition and Analysis System" Technical Report No. 1063.

2. Joshua Lederberg, "DENDRAL, A System for Computer Construction, Enumeration and Notation of Organic Molecules as Tree Structures and Cyclic Graphs", Part III, Complete Chemical Graphs; Embedding Rings in Trees.

3. "Molecular Biology Applications of Mass Spectrometry", Final Report for Air Force Office of Scientific Research, Contract AF 49(638)-1599, (Technical Report No. IRL 1073).

   (This report covers work closely related to research carried out under the NASA grant.)

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